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     (FILE 'HOME' ENTERED AT 12:49:47 ON 25 SEP 2002)

     FILE 'MEDLINE, BIOSIS, CAPLUS' ENTERED AT 12:52:35 ON 25 SEP 2002
L1         36632 S FERTILIZ? (10A) IN VITRO
L2          1587 S FERTILIZ? (10A) IN VITRO (10A) (EGG OR EGGS)
L3            23 S L2 (P) (CHICK? OR FOWL OR HEN OR HENS OR AVIAN OR AVIANS)
L4          2085 S FERTILIZ? (20A) IN VITRO (20A) (EGG OR EGGS)
L5            35 S L4 (P) (CHICK? OR FOWL OR HEN OR HENS OR AVIAN OR AVIANS)
L6            12 S L5 NOT L3
L7             8 DUP REM L6 (4 DUPLICATES REMOVED)
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egg

A structure which the females of certain animal species lay as a means of reproduction, it contains a fertilized zygote and nutrition in the form of yolk for the developing offspring, sometimes contains other substances (e.g., the white of a hen's egg), sometimes surrounded by a protective outer shell.

(09 Oct 1997)

Previous: eflornithine hydrochloride, eft, EGD, egersis, egest, egesta, EGF, EGF like domain
Next: egg albumin, eggar, egg-bird, egg cell, egg cluster, egg coat

ovum

1. The female reproductive cell which, after fertilization, develops into a new member of the same species (von Baer, 1827), an egg.

2. The human ovum: a round cell about 0.1 mm. In diameter, produced in the ovary, where there is deposited around it a noncellular covering (oolemma, zona pellucida, zona radiata). It consists of protoplasm which contains some yolk, enclosed by a thin cell wall (vitelline membrane). There is a large nucleus (germinal vesicle), within which is a nucleolus (germinal spot). By extension, the word is also used to designate any early stage of the conceptus, when the embryo itself constitutes a tiny and insignificant part of the whole.

(18 Nov 1997)

Previous: ovulist, ovulite, ovulocyclic, ovulocyclic porphyria, ovulode, ovulum
Next: ovum implantation, ovum implantation, delayed, ovum transport, owe

Published at the Dept. of Medical Oncology, University of Newcastle upon Tyne

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From: Wilson, Michael
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To: STIC-ILL

TI Using the amniotic cavity of the developing chick embryo for the in vivo culture of early-stage mammalian embryos.
AU Blakewood E G; Jaynes J M; Johnson W A; Godke R A
SO POULTRY SCIENCE, (1989 Dec) 68 (12) 1695-702.

AGL

TI Cardiac teratogenicity of dichloroethylene in a chick model.
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SO PEDIATRIC RESEARCH, (1992 Jul) 32 (1) 23-6.

TI Production of transgenic mice from in vitro fertilized eggs cryopreserved by ultrarapid freezing.
AU Anzai M; Nakagata N; Matsumoto K; Ishikawa T; Takahashi Y; Miyata K
SO JIKKEN DOBUTSU. EXPERIMENTAL ANIMALS, (1994 Jul) 43 (3) 445-8.

TI Characterization and application of an avian in vitro spermatozoa-egg interaction assay using the inner perivitelline layer from laid chicken eggs
AU Robertson, L.; Brown, H. L.; Staines, H. J.; Wishart, G. J.
SO Journal of Reproduction and Fertility (1997), 110(2), 205-211

TI In vitro sperm binding, penetration and fertilization of recently oviposited chicken eggs.
AU Johnston, S.; Birrenkott, G.
SO Poultry Science, (1998) Vol. 77, No. SUPPL. 1, pp. 142.
Meeting Info.: Nineteenth Annual Meeting of the Southern Poultry Science Society and the 39th Annual Meeting of the Southern Conference on Avian Diseases Atlanta, Georgia, USA January 19-20, 1998
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—— Note ——

Production of Transgenic Mice from *In Vitro* Fertilized Eggs Cryopreserved by Ultrarapid Freezing

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(Received 7 December 1993/Accepted 4 March 1994)

In vitro fertilized mouse eggs (C57BL/6N), followed by ultrarapid freezing were used for production of transgenic mice by microinjection of the chicken β-actin promoter-driven the firefly luciferase cDNA (β act-Luc). Following micromanipulation, the survival rates of the cryopreserved eggs and of the fresh *in vitro* fertilized eggs (control) were 70.8% (131/185) and 71.9% (159/221), respectively. After transferring them into oviducts of psudopregnant recipients on Day 1, 13.6% (17/125) of the cryopreserved eggs developed to live offspring and 14.1% (21/149) of fresh eggs did so. It was confirmed by Southern blotting analysis that each two transgenic mice were produced from the cryopreserved eggs (12%, 2/17) and the fresh eggs (10%, 2/21). All of transgenic mice produced from both eggs showed the expression of the luciferase gene. These results indicate that the *in vitro* fertilized eggs cryopreserved by ultrarapid freezing, can be, easily and conveniently, used for generation of transgenic mice.——KEY WORDS : mouse pronuclear oocyte, transgenic mouse, ultrarapid freezing

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超急速法で凍結した体外受精由来前核期受精卵を用いたトランスジェニックマウスの作製

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現在，遺伝子の顕微注入法によるトランスジェニックマウスの作製には，大量の前核期受精卵を必要とし，実験ごとに過排卵処理を施した供試卵用マウスから直接もしくは体外受精によって得た新鮮な前核期受精卵を供試卵としている。そのため供試卵用マウスや交配用雄マウスを数多く用意し，さらには胚移植のために成熟雌および精管結紮雄マウス等も常備していなければならず，限られた飼育スペースのなかでこれらマウスを長期に管理していくことは，多大な労力と経費を要する。

1991年 Leibo ら [3] は，このようなトランスジェニックマウスの作製をより効率化する1つの方法として，緩慢凍結法によって凍結保存して得た凍結融解卵子に遺伝子注入する方法を検討したところ，導入遺伝子の組み込まれたマウスが得られたことを報告している。一方，すでに著者らは，超急速凍結法を用いてマウス

の前核期受精卵，2・4細胞期胚および桑実胚あるいは未受精卵の凍結を行ったところ，良好な生存率が得られたことを確認している[5,6,7,8,9,10]。本実験では，より効率的なトランスジェニックマウスの作製方法の確立を目的に，緩慢凍結法に比べ簡易かつ短時間で操作の完了する超急速凍結法にて体外受精由来前核期受精卵の凍結保存を行い，凍結融解後のそれら受精卵を用いてトランスジェニックマウスの作製を試みた。

体外受精：過排卵誘起および体外受精は，豊田らの方法[14]に準じて行った。即ち，C57BL/6NJcl 雌マウス（8週齢：日本クレア）に5IU の PMSG と5IU の hCG を48時間間隔で腹腔内投与して過排卵処置を施し，hCG 投与後15～16時間に卵管膨大部から未受精卵を採取した。また，同系統の成熟雄マウス（9週齢：日本クレア）の精巣上体尾部より採取した精子塊を，HTF 培地[12]内で懸濁し，約1.5時間前培養後，先に採取した未受精卵と体外受精を行なった。媒精後約6時間目に倒立顕微鏡下で第2極体の放出と雌雄前核の形成を確認した前核期受精卵を新鮮な HTF 培地で洗浄後，凍結保存に使用した。

前核期受精卵の凍結および融解操作：凍結保存・融解操作は中潟[5,9]により既にマウスで確立されている超急速凍結法を用いた。受精卵の凍結は，受精卵（30～40個程度）を少量の培地と共に直接サンプリングチューブ内の20－30$\mu\ell$の高濃度の保存液 DAP213 (2M Dimethylsulfoxide, 1M acetamide, 3 M Propyleneglycol in PB1) に移し，すばやく液体窒素中に浸漬することにより超急速的に行った。融解操作は，凍結したサンプリングチューブを液体窒素保管器から取りだし，37℃の温水中で0.3mℓの0.3M sucrose (in PB1) 溶液をすばやくサンプリングチューブ内へ加え，受精卵を融解と同時に保存液を希釈，更にもう一度0.3mℓの0.3M sucrose 溶液でサンプリングチューブ内を洗い卵子を回収した。回収された卵子は新鮮な HTF 培地で3回洗浄した後，それらの形態学的観察を行ない，形態的に正常と判断された卵子については炭酸ガス培養器（37℃，5％CO_2，in air）内で約1時間静置後，遺伝子の顕微注入実験に供した。

トランスジェニックマウスの作製：受精卵への導入遺伝子（βact-Luc）[4]の注入操作法および胚移植は，既法[2]に従い行った。即ち，HTF 培地内で支持ピペットで卵子を保定後，あらかじめ DNA 溶液（4 μg/mℓ）を満たした注入ピペットを雄性前核に刺し DNA 溶液約2 pl を注入した。注入後形態的に正常な卵子については，精管結紮雄との交配によって誘起された偽妊娠第1日目の Jcl：MCH (ICR) 雌（12週齢：日本クレア）の卵管内に移植し，新生児への発生について検討した。また，得られた産子における導入遺伝子の染色体への組み込みについては，尾部組織から抽出した高分子 DNA よりサザンブロットハイブリダイゼーション法[13]で確認した。また，体外受精後，凍結保存を行わない卵子即ち，媒精後約6時間目に卵子を回収し HTF 培地で洗浄後，雌雄両前核形成の明瞭な卵子を対照区（unfrozen）として，トランスジェニックマウス作製に供した。

Table 1. Injection of β act-Luc fusion gene into cryopreserved *in vitro* pronuclear oocytes

Groups	No. of oocytes (%)	
	Used	Injected successfully
Unfrozen (Control)	221	159 (71.9)
Frozen-thawed	185	131 (70.8)

トランスジェニックマウスの解析：導入したルシフェラーゼ遺伝子の個体における発現は，ピッカジーン発光キット (PGK-L100，東洋インキ㈱) を用いて測定した。即ち，脳組織より抽出用バッファー（100mM KH_2PO_4，1mM DDT，pH 7.5）でホモジナイズして得られた細胞抽出液20$\mu\ell$をルミノメーター（ラボサイエンス，TD4000）にセットした後，発光基質100$\mu\ell$を注入することにより発光させ，30秒間その発光量を測定した。尚，ルシフェラーゼ活性は，ピーク時の発光量を BIO-RAD PROTEIN ASSAY (No.500-0001, Bio-Rad. Lab.) で測定した各抽出液中の総蛋白質量で補正することで得られた。

C57BL/6N を用いて体外受精を行なった結果，約90％（凍結区，447/496；対照区，221/242）の卵子が前核期受精卵へ発生した。また，凍結区においては遺伝子注入操作を行なうため一部の卵子を融解した結果，90%以上 (226/234) が回収され，回収した卵子の80%以上（185/226）が形態的に正常であった。

βact-Luc 遺伝子を融解した凍結受精卵へ注入後の卵子の生存性を Table 1. に示す。凍結融解後形態的に正常な卵子185個に遺伝子注入操作を行った結果，供試卵の70.8%（131/185）が注入後生存した。新鮮卵を使った対照区では，顕微注入操作を施した221個中その71.9%に当たる159個の卵子が生存していた。

Table 2. には，遺伝子注入操作をした卵子の移植後

Table 2. Production of transgenic mice from unfrozen and frozen pronuclear oocytes

Groups	No. of oocytes transfered	No. of recipients		No. of offspring (%)	No. of mice analyzed	No. of transgenic mice(%)		
		Used	Pregnant			Total(%)	♀	♂
Unfrozen(Control)	149	6	6	21(14.1)	21	2(9.5)	1	1
Frozen-thawed	125	7	5	17(13.6)	17	2(11.8)	0	2

の新生児への発生能およびトランスジェニックマウスの作製結果を示した。凍結区では，移植可能な卵子125個を偽妊娠第1日目のレシピエント雌に移植し13.6%（17/125）の卵子が新生児へ発生した。また，得られた全ての産子の尾部組織から抽出した高分子DNAをサザンブロッティング法で解析した結果，2匹(2/17：11.8%）のマウスに導入遺伝子の組み込みが確認された。

一方，対照区では，移植可能な卵子149個をレシピエント雌へ移植した結果，21匹（21/149：14.1%）の産子が得られ，同様にサザンブロッティング法で解析を行ない，2匹 (2/21：9.5%) のマウスに導入遺伝子の組み込みが確認された。尚，遺伝子注入操作後の卵子の生存率，移植成績および得られた産子における遺伝子導入動物数の割合は，いずれにおいても凍結区と対照区の両者間に有意差は認められなかった ($P>0.05$)。

対照区および凍結区のトランスジェニックマウスの脳組織より細胞抽出液を得て，そのルシフェラーゼ活性を検出したところ，全てのマウスにルシフェラーゼ遺伝子の発現が認められた（Fig 1.)。

本実験結果から，超急速凍結法によって凍結保存した体外受精由来前核期卵に遺伝子を顕微注入することにより，トランスジェニックマウスの作製が可能であることが示唆された。Leiboら [3] は，緩慢凍結法で凍結保存した過排卵体内受精卵を供試卵とした場合，新生児への発生率は新鮮卵（対照区）に比べ低いものの，トランスジェニックマウスの作製効率（移植した受精卵に対する作製された遺伝子導入動物数の割合）は，ほぼ同じであることを報告している。超急速法にて凍結した卵子を用いた本実験では，凍結区と対照区間において，遺伝子注入操作後の卵子の生存率，移植成績，得られた産子数に対する遺伝子導入動物数の割合および導入遺伝子の発現率に差が認められなかった。また，移植卵子に対するトランスジェニックマウスの作製率も凍結区で1.6%，対照区で1.3%とほぼ同様の値であり，さらに，この結果は，Brinsterら [1] の報告したトランスジェニックマウスの作製率（1～2%）と一致していることから超急速法により凍結した前核期卵は，トランスジェニックマウスの作製に利用できると考えられる。

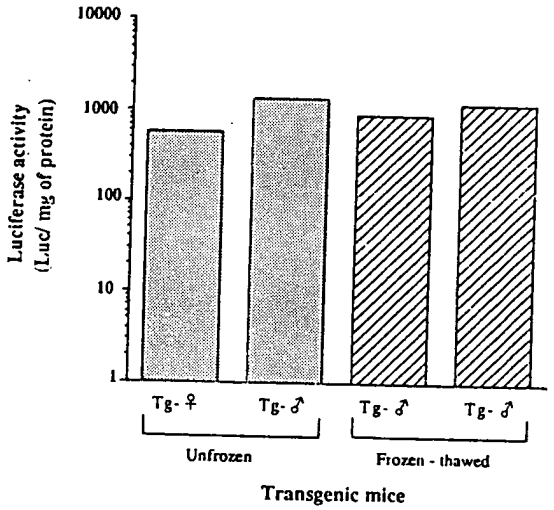

Fig. 1. Expression of luciferase gene in brain tissues of transgenic mice

マウス前核期受精卵の凍結保存について，緩慢凍結法の場合，凍結融解後の生存率および新生児への発生率は，低いことが報告されている [11,15] が，超急速凍結法を用いた F_1 (C57BL/6J×C3H) マウス前核期受精卵の凍結保存においては，緩慢凍結法の結果と比べ高い生存率が得られている [7]。C57BL/6N 前核期受精卵を用いた本実験においても，融解後の回収胚に対する形態的に正常な卵子の割合は80%以上と高率であったこと，更に，遺伝子注入以降の操作に対する生存性に関しても対照区と比較し，なんら差を認めなかったことから，前核期受精卵の凍結保存に，超急速凍結法は有効であることが再確認された。

現在，トランスジェニックマウスの作製における遺伝子注入操作には，新鮮な受精卵が用いられており，遺伝子注入の度に雌に過排卵処理を行い雄と交配するかあるいは体外受精によって，前核期受精卵を作出している。したがって，あらかじめ受精卵を凍結保存しておけば，実験当日に移植される受容雌の数に応じてそれら凍結卵子を融解できることから，遺伝子注入操作から遺伝子注入卵の移植までの一連の実験操作が，効率的に遂行され得ると思われる。また，実験計画が立て易く，実験日の前日迄なら臨機応変に実験日の変更が可能である。更に，著者らが開発した超急速凍結法は，緩慢凍結法に比べて極めて簡易であり，融解も5分以内で完了することから，トランスジェニックマウスの作製に用いる受精卵を短時間に準備できる。以上のことから，今後，超急速法により凍結した受精卵をトランスジェニックマウスの作製に応用することができれば，実験に費やす時間の大幅な短縮と実験そのものの簡略化が可能となろう。

要　約

トランスジェニックマウスの作製をより効率化することを目的に，超急速凍結法で保存した体外受精由来前核期受精卵を用いてトランスジェニックマウスの作製を試みた。体外受精によって得られた前核期受精卵（C57BL/6N）を超急速凍結し，融解後の卵子に遺伝子（βact-Luc）を顕微注入した。また，対照区には新鮮な体外受精卵を用いた。注入後，それぞれ70.8%（131/185），71.9%（159/221）の卵子が生存し，それらを偽妊娠第1日目のレシピエント雌卵管内に移植した結果，凍結区で13.6%（17/125），対照区で14.1%（21/149）の産子を得た。更にサザンブロット解析により，いずれの区においても2匹に導入遺伝子の組み込みを認め（凍結区，2/17：12%；対照区，2/21：10%），また，各トランスジェニックマウスの脳組織についてルシフェラーゼ活性を調べた結果，全てのマウスで遺伝子の発現を確認した。以上の結果より，超急速凍結法で凍結保存した体外受精由来前核期受精卵よりトランスジェニックマウスの作製が可能であることが示された。

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Main / MISF 481. P6

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AU Robertson, L.; Brown, H. L.; Staines, H. J.; Wishart, G. J.
SO Journal of Reproduction and Fertility (1997), 110(2), 205-211

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Using the Amniotic Cavity of the Developing Chick Embryo for the *In Vivo* Culture of Early-Stage Mammalian Embryos[1,2]

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ABSTRACT The fertile chicken egg may provide an effective, inexpensive method for promoting the development of early-stage embryos from other species. Presently, the loss of viability associated with the *in vitro* culture of mammalian embryos is hindering the use of *in vitro* fertilization with farm animals. Consequently, alternative *in vitro* laboratory methods are needed for the culture of mammalian embryos. A new method has been developed that involves the culture of mammalian embryos in the amniotic cavity of a developing chick embryo. Chick embryos were placed into shell-less incubation (37 C) at the 72-h developmental stage. After 24 h of shell-less incubation, agarose-embedded mammalian embryos were injected into the amniotic cavity of the chick embryo. The mammalian embryos were first placed into a drop of liquid agarose. One to four embryos were then aspirated into a beveled injection pipette and cooled, allowing the agarose to harden. Following penetration of the amnion with the beveled pipette, the agarose cylinder containing the embryos was expelled into the amniotic cavity. The shell-less culture system was then returned to incubation at 37 C for an additional 72 to 96 h. Following incubation, the amniotic cavity containing both chick and mammalian embryos was isolated and the agarose-embedded mammalian embryos were harvested. Significantly more embryos developed in the chick embryo amnion than in the control medium alone. Results obtained using this method on laboratory animals (mice) and on domestic mammals (goats and cattle) indicate that the chick-embryo amnion can support the development of early-stage, mammalian embryos to the blastocyst stage of development. This unique approach may be an alternative to existing systems for culturing the embryo *in vivo* and *in vitro*.

(*Key words*: mammalian embryos, chick embryos, viability, shell-less culture)

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INTRODUCTION

The apparent inability of defined culture media to support the growth of early mammalian embryos to the blastocyst stage has resulted in the development of several, alternate culture techniques. These include *in vitro* coculture with feeder cells (Cole and Paul, 1965), fibroblast monolayers (Kuzan and Wright, 1982; Voelkel *et al.*, 1985) or trophoblastic vesicles (Camous *et al.*, 1984; Heyman *et al.*, 1987; Pool *et al.*, 1988), and *in vivo* culture in the ligated oviducts of sheep (Willadsen, 1979). Recent successes in producing *in vitro*-fertilized bovine embryos (Lu *et al.*, 1987), pronuclear gene injection (Hammer *et al.*, 1985), and nuclear transplantation (Willadsen, 1986; Robl *et al.*, 1987) have increased the importance of early embryo culture. The purpose of this study was to develop a technique that would allow early-stage mammalian embryos to be introduced into the amniotic cavity of a developing chick embryo and subsequently recovered following 72 to 96 h of culture.

Chick embryo extract (CEE) was among the first factors used to stimulate the growth of mammalian cells in culture. Carrel (1913) found that the CEE increased the growth of canine connective tissue at least three-fold during *in vitro* culture. In later studies with avian tissues, Willmer and Jacoby (1936) showed that CEE had the ability to stimulate the development of cells that had ceased to grow in culture, with the rate of proliferation proportional to the concentration of CEE. New and Stein (1964) cultured postimplantation mouse embryos on plasma clots containing either mouse-embryo extract or CEE; they noted that CEE was capable of promoting the

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in vitro growth of mouse embryos at a rate similar to that of mouse-embryo extract.

Recently, human amniotic fluids have been used as an alternative to balanced salt solutions for human *in vitro* fertilization (Gianaroli *et al.*, 1986). The amniotic fluids used in that study were collected from women during different stages of gestation. All such fluids were capable of supporting the growth of two-cell, murine embryos at a rate similar to that of a serum-supplemented medium. Furthermore, pregnancies were obtained from *in vitro*-fertilized human embryos cultured in these human amniotic fluids. Recently, embryotropic activity has been verified in human amniotic fluids using early-stage murine embryos cultured with amniotic fluids obtained from women during early pregnancy (Ball *et al.*, 1988). These embryotropic properties may also exist in the amniotic fluids of chickens.

MATERIALS AND METHODS

Preparation of In Vitro *Chick Embryos.* Fertilized chicken eggs were collected from domestic White Leghorn hens on the day of oviposition and stored at 10 C until incubation. Four days prior to the collection of mammalian embryos, the eggs were removed from cold storage, rinsed with a solution of 70% ethanol, and placed in a 37.5 C commercial egg incubator (75 cm square) which automatically rotated the eggs 60° every 60 min to prevent the adhesion of egg contents to the shell membrane. After 72 h of incubation, the eggs were rinsed again with a solution of 70% ethanol and allowed to air-dry. Then, the eggs were coated with a commercial Betadine solution (Purdue Fredrick Company, Norwald, CT) and allowed to dry in a horizontal position, thus preparing for an appropriate positioning of the chick embryo. All subsequent preparation of shell-less cultures was performed under a laminar-flow hood in order to minimize the potential for contamination.

The original procedure for the shell-less incubation of developing chick embryos (Dunn, 1974; Dunn and Boone, 1976) was modified as follows for use as a mammalian-embryo coculture system. To remove the egg contents, two pieces of medical adhesive tape stretched across the jaws of a pair of 200-mm, surgical retractors were used to affix the egg in place at each pole. A crack was made by striking the area of shell between the pieces of tape against the rim of a sterile beaker. The cracked egg was then held above a piece of cellophane kitchen wrap (Saran®, Dow Chemical, Midland, MI), which covered a 100-mL plastic embryo collection bowl (Veterinary Concepts, Spring Valley, WI). The egg contents were released by opening the spreaders. The cellophane wrap was folded around the rim of the collection bowl, and any excess was trimmed away. Then, the bowl with the egg contents was placed in a second bowl to secure the cellophane wrap and was loosely capped with a plastic lid (Veterinary Concepts, Spring Valley, WI). This shell-less culture system was then maintained in an incubator at 37 C with an atmosphere of 2% CO_2 in air for an additional 24 h prior to introducing the mammalian embryos.

Injection of Agarose-Embedded Embryos into the Chick Amnion. The procedure for embedding early-stage embryos in agarose was modified from that used by Willadsen (1979) for micromanipulated ovine embryos prior to culture in the ligated oviducts of sheep. Because recovery rates of nonembedded embryos from the chick embryo amnion were typically less than 50% during a preliminary study, agarose-embedding was used in the present experiment in an attempt to increase embryo recovery rates. Low-melting-point, electrophoresis-grade agarose (Catalog No. 5517UA, Bethesda Research Laboratories, Gathersburg, MD) was added to 1 mL of sterile Dulbecco's phosphate-buffered saline (PBS: Gibco, Grand Island, NY) (pH 7.2, 270 mOsm) at a concentration of 1.5% and dissolved by warming to 75 C for 10 min. Dissolved agarose was filtered through a .2-μm acrodisc and cooled to 50 C, then 10 μL of an antibiotic-antimycotic solution containing 100 units of penicillin, 100 μg of streptomycin, and .25 μg of amphotericin-B (Gibco, Grand Island, NY) was added. The agarose solution was maintained in a water bath at 50 C.

Immediately prior to embedding, a 50-μL drop of agarose was pipetted into a sterile petri dish and moved to a warming hood where the embryos were held in one of either two media at 35 C: 1) N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-buffered Whitten's medium (murine embryos); or 2) a PBS holding medium (bovine and caprine embryos). One to four embryos were transferred to each 50-μL agarose droplet. As

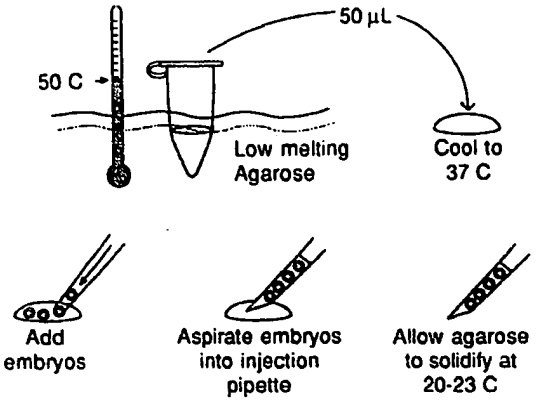

FIGURE 1. Procedure using a beveled, injection pipette for agarose-embedding of mammalian embryos.

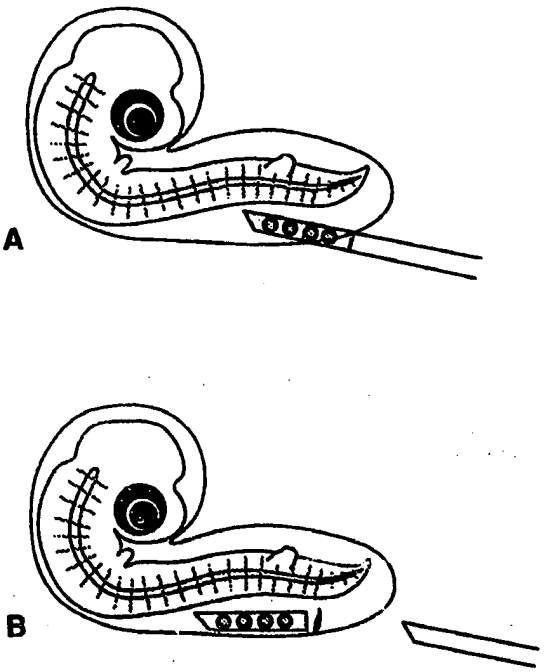

FIGURE 2. Procedure for placing mammalian embryos in the chick-embryo amniotic cavity. Penetration of 96-h chick amnion with a beveled injection pipette (A) and removal of the pipette following injection of agarose-embedded mammalian embryos (B).

the embryos were added, the droplets were stirred to dilute the holding medium in the agarose. The embryos in the agarose solution were then aspirated into a beveled glass pipette (200 µm, inside diameter) and cooled to 25 C for 1 min to allow the agarose to gel (Figure 1).

Pipettes were prepared by heating borosilicate, glass capillary tubes [1 mm, outside diameter (o.d.)] over a microburner and drawing them out to an o.d. of 250 µm. The pipettes were beveled on a microgrinder at an angle of 45° to enable a controlled puncture of the chick amniotic membrane. An *in vitro* chick embryo at the appropriate developmental stage for injection (96 h of incubation) was removed from the incubator as the agarose began to congeal. The lid was removed from the bowl containing the shell-less, chick embryo under a warming hood; and the amniotic membrane was visualized (10 power) using a Zeiss stereomicroscope (Model M-3, Thornwood, NY) equipped with an overhead light source. The amniotic membrane was manually pierced using the beveled pipette containing agarose-embedded embryos. When the amniotic membrane had been punctured, the embedded embryos were expelled from the pipette; and the pipette was carefully withdrawn (Figure 2). After introducing the embedded embryos into the amniotic cavity, the *in vitro* chick embryos were incubated an additional 72 to 96 h at 37 C in an atmosphere of 2% CO_2 in air.

Mammalian Embryo Recovery. The *in vitro* chick embryos were individually removed from incubation after 72 h of mammalian embryo (murine) coculture, and the plastic lids were removed. The posterior portion of the chorioallantoic membrane was carefully lifted and drawn anteriorly over the chick embryo. A sterile, plastic spoon was then used to gently lift the chick and amnion from the rest of the egg contents. Extra embryonic membranes were trimmed away using a pair of surgical scissors. Next, the amnion containing the chick embryo was placed in a sterile petri dish and rinsed twice with 5 mL of the PBS solution with 1% heat-treated fetal bovine serum (FBS) and 1% Gibco antibiotic-antimycotic solution (Ab-Am). After rinsing, 5 mL of the PBS solution was again added to the chick embryo; and the petri dish was moved to a stereomicroscope. Agarose cylinders containing the mammalian embryos could be seen within the amniotic cavity by using a stereomicroscope at 10-power magnification. The agarose cylinder was removed from the amniotic cavity by

using two hypodermic needles (22-gauge, beveled) to manually make a small opening (1 to 2 mm) in the amniotic membrane near the agarose cylinder. The escaping amniotic fluids then carried the agarose cylinder out into the surrounding PBS medium. When the agarose cylinder settled in the petri dish, the mammalian embryos were carefully extracted from the agarose using the same two hypodermic needles. The embryos were then rinsed in the PBS holding medium prior to further culture.

Experimental Design. Donor mice (21-day C57/J and F_1 SJL/J × C57 females) were superovulated with 5 IU of pregnant mare serum gonadotropin, followed at 48 h with 5 IU of human chorionic gonadotropin (hCG) and placed with either C57 or SJL/J males. The F_1 females were crossed back to SJL/J males since SJL/J embryos characteristically show a two-cell, *in vitro* developmental block. The cumulus cells were removed from the embryos (collected 26 h after HCG) by treatment with 150 IU/mL of hyaluronidase in HEPES-buffered Whitten's medium (Whitten and Biggers, 1968).

The embryos were evaluated for the presence of pronuclei, then randomly and equally allotted by parental strain to the treatment group or the control group. The embryos in Treatment 1 were embedded in agarose cylinders and injected into the amniotic cavity of a chicken embryo that had been incubated for 96 h. Following injection, the culture system was placed in a 37 C incubator containing 2% CO_2 in air. The control embryos (Treatment 2) were cultured in Whitten's medium with 1% Ab-Am in a 5% CO_2 incubator at 37 C. After 72 h of culture, the agarose cylinders were recovered from the chick amniotic cavity, and the embryos (late morula-to-blastocyst stage) were carefully removed from each cylinder. Embryos from Treatments 1 and 2 were then placed in Ham's F-10 medium (Gibco, Grand Island, NY) with 10% FBS and 1% Ab-Am (HF-10) and were cultured at 37 C in an atmosphere of 5% CO_2 in air for an additional 48 h to evaluate *in vitro* development.

Typically, some shell-less chick embryos maintained in incubation will not remain viable (Rowlett and Simkiss, 1987). So, like-quality, pronuclear mouse embryos (collected from corresponding donor females) were used to replace the embryos lost if chick embryo mortality occurred during the coculture period in Treatment 1. Likewise, if the individual culture wells used for the medium-only group (Treatment 2) became contaminated, embryos of similar quality were used for replacement. This was done to evaluate the growth and development of the mammalian embryos across functional culture systems.

Precompaction-stage bovine morulae (8 to 16 cells) were nonsurgically collected from superovulated, crossbred, beef donor cattle on Days 5 or 6 postmating (the onset of estrus equals Day 0 of the estrous cycle). Bovine embryos were randomly allotted by embryo quality grade and donor female either to the chick embryo coculture or the HF-10 culture (control). Embryos cocultured with chick embryos were agarose-embedded and were injected into the chick amnion, the same as with murine embryos. (See the previous description.) After 72 h in the chick amniotic cavity, the embryos were placed in the HF-10 medium for further culture. Goat embryos (2 to 8 cells) were surgically collected from the oviducts of superovulated does 36 h postestrus and were cocultured with chick embryos in two further studies.

In the first experiment, the 72-h chick-embryo coculture was compared with the HF-10 culture (control). In the second experiment, the duration of the chick-embryo coculture was increased to 96 h and was compared with the HF-10 culture (control). In each case, bovine and caprine embryos were further cultured in HF-10 following recovery from the chick amnion to evaluate *in vitro* development.

Statistical Analysis. A chi-square analysis was used to compare the mean number of mammalian embryos with complete blastocoele-cavity development (expanded blastocyst) and the mean number of embryos emerging from an opening in the zona pellucida (hatching blastocyst) between treatment groups (Treatments 1 and 2) during and following culture.

RESULTS

Of the 100, pronuclear mouse embryos embedded and placed in the amniotic cavities of chick embryos, 84% were successfully recovered for culture in the HF-10 medium. The loss of embryos either occurred during the recovery process or was due to chick-embryo mortality. Five of the 35 chick embryos (14%) originally injected did not survive shell-less incubation, accounting for the majority of the

TABLE 1. *The number and percentage of murine blastocysts that developed following culture in the chick-amnion or Whitten's control medium*

Parental strain	Treatment	(n)	Expanded blastocysts		Hatching blastocysts	
			(n)	(%)	(n)	(%)
C57/J × C57/J	1	42	38	90	24	57[A]
	2 (control)	42	33	78	11	26[B]
F_1 (SJL/J × C57) × SJL/J	1	42	39	93[A]	27	64[A]
	2 (control)	42	15	36[B]	4	10[B]

[A,B]Values within columns and strains with no common superscripts were different (P<.01).

embryo loss. Chick-embryo mortality tended to be distributed evenly over the 72-h culture period. Sixteen embryos were replaced in Treatment 1 in order to maintain an equal number of embryos for culture comparisons across experimental groups. Since no culture wells were contaminated during incubation, no embryos in Treatment 2 were replaced.

Overall, significantly more hatched blastocysts resulted from mouse embryos cultured in the chick-embryo culture system both within strains of mice and overall for the treatments, compared with those from the control culture system (P<.01) (Table 1).

In the present study, more hatched blastocysts were obtained from both strains of mouse embryos when cultured for 72 h in the chick-embryo amnion (Treatment 1) than from those hatched by the control embryos (Treatment 2). For Treatment 1, the comparative data were 57% (24/42) and 64% (27/42), respectively, hatched among C57 × C57 and F_1 (SJL/J × C57) × SJL/J embryos; for Treatment 2, 26% (11/42) and 10% (4/42), respectively.

There was no significant difference in the number of expanded blastocysts in culture obtained by using the C57/J embryos: 90% (38/42) of the chick-embryo treatment (Treatment 1) were expanded; 78% (33/42) of the Whitten's-medium treatment (Treatment 2) were expanded. Among the F_1 (SJL/J × C57) × SJL/J embryos, however, significantly more expanded blastocysts were obtained by using the chick-embryo coculture: 93% (39/42) of those in Treatment 1 (chick-embryo treatment) were expanded, compared with 36% (15/42) of those in Treatment 2 (Whitten's control medium).

Among the bovine precompaction-stage morulae, there were significantly more expanded and hatching blastocysts in the chick-embryo treatment versus the HF-10 treatment: 80% (16/20) expanded and 35% (7/20) hatching blastocysts with the chick-embryo treatment, compared with 15% (3/20) and 0% (0/20) with the HF-10 (control) treatment.

The goat embryos (2 to 8 cells) cocultured in the chick amniotic cavity also reached the expanded and hatching blastocyst stage in significantly greater numbers than did the embryos cultured in the HF-10 medium alone. None of the oviductal-stage goat embryos (Experiment 1 equals 0/20, Experiment 2 equals 0/22) developed into blastocysts in the control culture medium alone. However, 70% (14/20) reached the expanded blastocyst stage, and 55% (11/20) hatched when cocultured with chick embryos for 72 h (Experiment 1). At 96 h of chick-embryo coculture (Experiment 2), 86% (19/22) of the embryos reached the expanded blastocyst stage and 82% (18/22) hatched during the *in vitro* culture interval.

DISCUSSION

The results obtained indicated that factors present in the amniotic cavity of developing chick embryos are capable of supporting the growth of early murine embryos. Since the living chick embryo actively regulates the pH, osmolarity, and O_2 content of its own environment, this may make it a more stable physiological system for mammalian embryo development than that provided by the existing, *in vitro* embryo culture systems. In addition, the active synthesis of growth factors associated with the chick embryo may provide a stimulus to growth and development not obtainable from conventional culture systems that require supplementation with active serum or other growth factors.

The growth factors associated with the highly regulated mechanisms of early chick-embryo development may also prove to be more consistent in their growth-promoting effects than those found in processed mamma-

lian serum. Sirard and Lambert (1985) have shown that identically prepared batches of bovine serum from different animals produced different results in terms of their ability to promote the cleavage of four-cell bovine embryos. Different batches of bovine serum albumin (BSA) from the same supplier have also been shown to have different growth promoting effects on mammalian embryos *in vitro*. Kane (1983) has reported that rabbit morulae cultured to the blastocyst stage in a medium supplemented with 1.5% BSA from one batch had more than twice as many cells as morulae cultured in the same medium supplemented with a different batch of BSA.

Water purity has been shown to be critical for the maintenance of embryo viability *in vitro*. Whittingham (1977) reported that three-times glass distilled water was necessary for a high rate of blastocyst formation from two-cell murine embryos. Abramczuk *et al.* (1977) showed a beneficial effect on the culture of one-cell murine embryos when EDTA as a chelating agent was added to the culture medium. These results suggest that even trace impurities can greatly impede the normal development of mammalian embryos in culture.

Gianaroli *et al.* (1986) considered human amniotic fluid to be an appropriate embryo culture medium since it is an ultrafiltrate produced in contact with the developing human fetus. Likewise, chick-embryo amniotic fluid may be an ultrafiltrate of the avian egg. If so, embryotoxic impurities may be absorbed and filtered by the egg membranes and chick tissues before reaching the amniotic fluid.

The shell-less, chick-embryo cultures in this study were maintained in an atmosphere of 2% CO_2, as described by Dunn and Boone (1976). More recent work involving the *in vitro* culture of shell-less chick embryos in the authors' laboratory indicates that normal development is possible at lower concentrations of CO_2 in atmospheric air. Rowlett and Simkiss (1987) have successfully "hatched" chicks cultured in atmospheric air by replacing the plastic bowl with a surrogate eggshell. The apparent ability of the *in vitro* chick embryo to grow in a warm, moist atmosphere that does not require careful monitoring of CO_2 concentrations may make chick-embryo coculture a viable option in situations where CO_2 incubators are not available.

In the present experiment, embryos were not recovered from chicks that died during the coculture interval. In previous trials, a rapid degeneration of mammalian embryos occurred following chick embryo death. At present, the inherent mortality of developing chick embryos is the primary drawback to using this procedure. The hatchability of intact, fertile chicken eggs during incubation has been reported to be 90%, with embryo mortality peaking at Days 3 and 18 of incubation (Guilbert, 1974). Correspondingly, Rowlett and Simkiss (1987) have previously reported a 27% loss of chick embryos between Days 4 and 7 when 362 shell-less, chick embryos were maintained in incubation.

In the present study, the mortality of chick embryos was 14%. This loss may be due, in part, to the manipulations associated with shell-less, chick-embryo culture. Although the exact cause of mammalian embryo death in the present experiment is not known, the decline in the viability of mammalian embryos noted after chick-embryo death does suggest that there was a high rate of transfer across the agarose cylinder containing the mammalian embryos. The frequent amniotic contractions observed in the healthy chick embryo (Romanoff, 1952) may have promoted an even distribution of nutrients, growth factors, and dissolved oxygen within the amniotic fluids surrounding the agarose cylinder.

The duration of chick-embryo coculture was limited to 72 h in the murine and bovine experiments in order to remove the mammalian embryos from the agarose cylinder before the hatching blastocyst stage. However, when the coculture was extended to 96 h for the early-stage caprine embryos, high embryo recovery rates (>90%) resulted. Even longer coculture intervals may be possible. Placing embryos in the amnion prior to Day 4 of incubation may not be feasible due to the fact that the chorioamniotic folds of the chick embryo are not closed until then. However, coculture begun at Day 4 probably can be continued well in excess of 96 h. Unfertilized ova have been agarose-embedded and injected into the amnion of 96-h chick embryos in order to test the feasibility of longer coculture periods. Successful recovery of these ova was possible after 120 h in the chick amniotic cavity. In theory, the use of the amniotic cavity as a culture vessel could continue until Day 11 or 12, when the chick begins to ingest the amniotic fluid. The practicality of recovery and the characteristics of the amniotic environment

need to be evaluated during these latter stages of embryo development.

The ability to consistently fertilize bovine embryos *in vitro* would allow researchers to obtain large numbers of early embryos from slaughterhouse ovaries. These embryos could then be used in gene-transfer procedures (Hammer *et al.*, 1985) and embryonic-cloning techniques (Willadsen, 1986; Robl *et al.*, 1987). Such procedures and techniques require embryos at a very early stage of their development. However, those same embryos would not be considered good candidates for immediate transfer since acceptable pregnancy rates following embryo transfers in cattle occurs only when the embryos had reached the morula and blastocyst stages of development (Schneider *et al.*, 1980; Massey and Oden, 1984; Hasler *et al.*, 1987). If early-stage embryos could then be cultured to a stage (morulae and blastocysts) which allowed them to be transferred nonsurgically to recipient females, significant gain could be made.

Currently, the loss of viability associated with the long-term, *in vitro* culture of early-stage embryos represents a weak link in this scenario. Consequently, as the *in vitro* fertilization of bovine embryos becomes more repeatable (Lu *et al.*, 1987, 1988), the need to culture early-stage embryos should increase markedly in the near future. If the unique, chick-embryo culture system developed in the present experiment were to prove to be at least as effective as *in vivo* culture in the ligated oviducts of sheep, the relative ease of chick-embryo coculture would make it an invaluable tool for research and for commercial applications.

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Episomal Replication of Cloned DNA Injected Into the Fertilised Ovum of the Hen, *Gallus domesticus*

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ABSTRACT We describe preliminary experiments to analyse the fate of cloned DNA microinjected into the cytoplasm of the chick fertilised ovum. The reporter gene construct pRSVcat was injected into the germinal disc before the first cleavage division, and the chick embryos were cultured for up to 7 days using the method of Perry (Nature 331:70–72, 1988). Linear plasmid molecules ligated rapidly after injection to form high-molecular-weight DNA molecules consisting mainly of random concatemers of the injected plasmid. Recombination involving circular molecules resulted in head-to-tail multimers of the plasmid. Some of the DNA was lost after injection, but the remainder was replicated approximately 20-fold during the first 24 h of development. Between days 1 and 7 in culture, the DNA was gradually lost and diluted out as the embryos developed. By day 7 in culture plasmid DNA was detectable in only 30% of the cultures analysed. No evidence for chromosomal integration of the exogenous DNA was obtained, suggesting that the plasmid DNA persisted episomally. Expression of the reporter gene construct pRSVcat was detected in day 2 and day 7 embryos.

Key Words: Concatemers, Ligation, pRSVcat, Recombination

INTRODUCTION

Injection of cloned DNA into ova or early embryos has been exploited in many systems to examine expression from the exogenous DNA or as a method to produce transgenic animals, with copies of cloned DNA stably integrated into the genome. In mammals the method of choice is to inject DNA directly into the pronucleus of the fertilised eggs. In mice this results in 10–15% transgenic animals among embryos surviving to term. The rate of production of transgenic mice is at least 10-fold lower if DNA is injected into the cytoplasm of the fertilised ovum (Brinster et al., 1985).

In species with large, yolky eggs, e.g., *Xenopus*, sea urchins, and fish, DNA injected into the cytoplasm can result in the production of transgenic animals but usually results in short-term episomal replication and persistence of the injected DNA. In *Xenopus*, injected DNA remains extrachromosomal during early cleavage stages. It is replicated up to blastula stage and then is gradually lost (Bendig, 1981; Rusconi and Schaffner, 1981). Germ line transmission of injected DNA by an adult male frog has been demonstrated by Etkin and Pearman (1987). In the sea urchin, cloned DNA injected into eggs, followed by fertilisation, replicates repeatedly during early development (McMahon et al,. 1985). Genomic integration of the cloned DNA has been observed (Flytzanis et al., 1985). Recent reports suggest that microinjection of DNA into fish oocyte cytoplasm can result in germ line transmission of the exogenous DNA (Chourrout et al., 1988).

In *Gallus domesticus*, efforts to develop a method to produce transgenic poultry, showing stable germ line transmission of inserted cloned genes, have concentrated on the use of retroviral vectors because injection of DNA at the single-cell stage was considered to be prohibitively difficult (Freeman and Messer, 1985). Manipulation of the chick zygote has been made possible by the development of a culture system for the chick fertilised ovum, described by Perry (1988). The ovum, which is the equivalent of the yolk in the laid egg, is fertilised shortly after ovulation. The male and female pronuclei are located in the germinal disc, the easily identifiable white spot lying on the surface of the yellow yolk. Fertilisation in birds is polyspermic, resulting in the presence of several morphologically identical paternal nuclei in the germinal disc (Perry, 1987). Direct injection of DNA into the pronuclei is impossible because they are masked by yolk spheres and because those destined to form the zygote nucleus cannot be distinguished from the supernumerary pronuclei. Here we describe preliminary experiments to investigate the results of injecting DNA into the chick

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germinal disc. The fate of cloned DNA injected at the single-cell stage followed by up to 7 days of embryo culture is analysed.

MATERIALS AND METHODS

Microinjection and Culture of Fertilised Ova

Hens of a commercial laying strain (Isa brown) at 28–32 weeks of age were artificially inseminated from Rhode Island red cockerels at weekly intervals. The hens were killed with sodium pentobarbitone (i.v.) (Expiral, Ceva Ltd) at 3 hr after oviposition. At this time the next ovum is usually located in the midposterior region of the magnum, and the pronuclei are swelling in preparation for syngamy some 15–60 min later (Perry, 1987). The ovum, encased in albumen, was removed from the oviduct and placed in a glass vessel with culture medium. Infertile ova, 24 hr post-ovulation, were obtained from laid eggs for one experiment.

DNA (1–5 nl, at concentrations of 10, 25, or 50 μg/ml in distilled water) or distilled water (1–5 nl) was injected into the cytoplasm near the centre of the germinal disc and at a depth of 150–200 μm beneath the vitelline membrane. The micropipettes were drawn from 1.5 mm capillary tubing with a pipette puller to produce uniformly tapered shafts 5.0 mm in length. The tips were bevelled down to a diameter of 10 μm. The shafts were calibrated at distances of 150–200 μm and 2.0–2.5 mm from the tips to determine the depth of penetration into the cytoplasm and the volume of medium, respectively. The micropipettes, one per injection, were connected to a 10 ml syringe with air-filled tubing (5 mm diameter) and were held in a micromanipulator (Microelectrode Implantation Unit, Research Instruments Ltd, Penryn, UK). They were loaded from the tip by capillarity and inserted through the albumen capsule and tough vitelline membrane into the germinal disc, with a final adjustment in position to display the lower calibration mark.

The manipulated embryos were cultured in sealed glass vessels (system I) for 24 hr at 42° C, then transferred to recipient egg shells (system II) for culture at 38° C over the following 6 days of development as described by Perry (1988). After 2 hr of incubation, the embryos had reached the 2–16-cell stage. At later times, the embryos were staged according to Hamburger and Hamilton's table of chick development (1951). The 24 hr cultures correspond to stage 1. Embryos for DNA analysis were taken at 24 hr intervals between 2 hr and 7 days of incubation. They were dissected from the yolk, rinsed in Dulbecco's phosphate-buffered saline without Ca^{2+} or Mg^{2+}, and stored at −80° C until required. For the younger cultures, up to 3 days of incubation, each embryo was taken as a single sample, or it was divided transversely into two samples. For older cultures, the embryo was divided into head, trunk and two separate samples of vascular extraembryonic membrane.

Plasmid DNA and Chloramphenicol Acetyltransferase (CAT) Assays

The plasmid pRSVcat (Gorman et al., 1982a) was provided by Dr. B. Howard. It contains the bacterial gene CAT coding sequences under control of the Rous sarcoma virus (RSV) long terminal repeat (LTR) promoter. CAT enzyme activity assays were performed as described by Gorman et al. (1982b). Tissue samples were disrupted by three freeze-thaw cycles in 200 μl of 0.25 M Tris HCl, pH 7.8, and 50 μl of extract was assayed for activity using ^{14}C-chloramphenicol. The results were analysed on silica gel thin-layer plates run with chloroform-methanol 95:5.

DNA Extraction and Southern Transfer Experiments

Embryo and blastoderm tissue samples were homogenised in 300 μl of urea lysis buffer (8 M urea, 0.35 M NaCl, 10 mM Tris HCl, pH 8, 10 mM EDTA); 10% Na-lauryl sarcosine solution was added to give a concentration of 1%, and the homogenate was incubated at 60° C for 1 hr. The homogenate was extracted twice with an equal volume of phenol (equilibrated with 10 mM Tris HCl, pH 7.4)/chloroform (1:1), and twice with an equal volume of chloroform. The total nucleic acid was precipitated with ethanol and redissolved in TE (10 mM Tris-HCl, pH 7.4, 1 mM EDTA). The nucleic acid was precipitated a second time and the dried pellet was redissolved in TE at 55° C overnight.

Restriction digestion of DNA was carried out according to manufacturers specifications. Submerged agarose gels were run in Tris acetate buffer (40 mM Tris, 20 mM Na acetate, 1 mM EDTA, pH 8.2) at ~1 V/cm. The DNA was transferred to nitrocellulose using the modification by Smith and Summers (1980) of the method of Southern (1975). Nick translated (Rigby et al., 1977) pRSVcat or the plasmid vector pUC9 was used as probe. Hind III-digested λ cI_{857} DNA was used as size markers, and 5 pg of linearised pRSVcat was loaded as a control for the level of signal detection in autoradiography of the Southern transfers. Filters were hybridised with at least 2×10^7 cpm labelled probe overnight at 37° C in 50% formamide, 4× SSC, 0.1% sodium dodecyl sulphate (SDS), 10× Denhardts, 3% dextran sulphate. Following hybridisation, filters were washed in 2× SSC, 0.1% SDS at room temperature for 2 hr, then in 1× SSC, 0.1% SDS at 37° C for 2 hr. Filters were exposed to preflashed X-ray film for 1 week.

RESULTS

Injection of DNA and Embryo Culture

DNA (25–150 pg) was injected into the cytoplasm of the germinal disc ~60 min before the first division of the zygote nucleus. Fertilised ova injected with water alone served as controls. The embryos were cultured for periods up to 7 days as described in Materials and

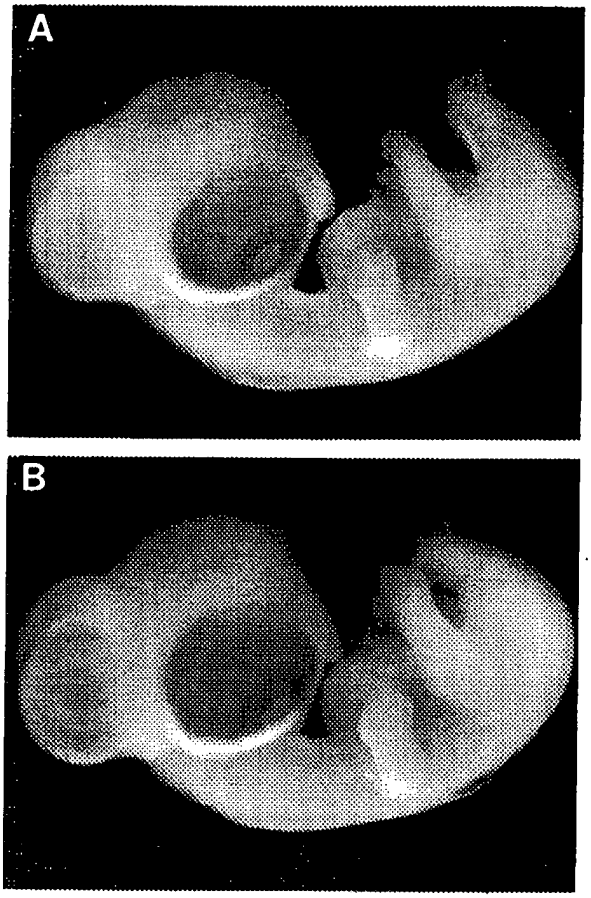

Fig. 1. A: Chick embryo (stage 29) from a fertilised ovum cultured in vitro for 1 day and in a recipient egg shell for 6.5 days. B: Chick embryo from a 6.5 day incubated egg.

Methods. The survival rate of uninjected embryos in culture is 60–70% at 7 days and zero at 8 days (Perry, 1988), the most advanced embryos corresponding in developmental age to those obtained from 6.5 day incubated eggs (Fig. 1). The survival rate was reduced to 40% or less in the manipulated control and experimental embryos cultured for 4 to 7 days following injection (Table 1). The developmental age of the surviving embryos was variable, the most advanced reaching the expected stage for the period of culture. In the remaining cultures, there was either a malformed embryo or an extensive outgrowth of cellular blastoderm or no evidence of growth. The material from the cultures incubated for longer than 4 days was retained for analysis only if the embryos appeared normal and were not grossly retarded. Preparations exhibiting abnormal development were also analysed from cultures incubated for less than 4 days. The procedure for each experiment listed in Table 2 was performed on a group of six to eight embryos at a time.

The plasmid DNA used in these experiments was

TABLE 1. Survival Rates of Chick Fertilised Ova Injected With pRSVcat and Incubated for 4–7 Days

Material injected	Number of ova injected	Number of viable embryos	Percent survival
pRSVcat (BamH I) 10 μg/ml	73	32	44
pRSVcat (BamH I) 25 μg/ml	129	48	37
pRSVcat (Nru I) 25 μg/ml	27	7	26
Water	34	13	38

pRSVcat (Gorman et al., 1982a) (shown in Fig. 2). It can easily be detected after injection, because it contains sequences foreign to the chick genome, and the activity of the bacterial CAT gene can be assayed to detect expression of the injected DNA. The fate of the injected DNA was assayed by extraction of total nucleic acid from the cultured embryos and analysis in Southern blotting experiments. Persistence of pRSVcat was detected by probing with labelled pRSVcat or, when analysing longer term cultures, with labelled plasmid vector only. pRSVcat contains sequences homologous to chick genomic sequences, which are detected when significant amounts of chicken DNA are present.

Linear DNA Is Ligated in Embryos After Injection

Ova were injected with pRSVcat linearised at the single BamH I site (Fig. 2A) and cultured for periods of 2 hr to 7 days. The number of experimental and control embryos, the range in amount of DNA injected, and the developmental stages reached after the given culture periods are summarised in Table 2, experiments 1–9. Total DNA extracted from each culture was analysed on agarose gels either intact or digested with the restriction enzyme Hind III, which cuts pRSVcat once (Fig. 2A).

Figure 3A shows the analysis of undigested DNA from infertile egg germinal discs, incubated for 2 hr at 41° C after injection (Table 2, experiment 1). The 5 kb monomeric pRSVcat is still clearly present, but a ladder of bands in 5 kb steps up to at least 20 kb can also be seen. This suggests that the linear DNA ligated in the germinal disc to form larger molecules. Fertilised ova were cultured for 2 hr and 24 hr after injection (Table 2, experiments 2 and 3). After 2 hr, there was evidence of ligation of the pRSVcat linear molecules. Undigested DNA from 24 hr cultures showed hybridisation to the plasmid vector probe, mainly at the limiting mobility of the gel (Fig. 2B). This demonstrates incorporation of the injected DNA into high-molecular-weight DNA. Injected ova were cultured for further periods of 2–7 days (Table 2, experiments 4–9) and analysed as described above. Plasmid sequences detected in Southern transfers comigrated with high-molecular-weight DNA (data not shown).

DNA samples from experiments 1–9 (Table 2) were

TABLE 2. Summary of Experiments on the Injection of pRSVcat Into Chick Fertilised Ova

Experiment	Culture period	No. of injected cultures analysed	Amount DNA injected (pg)	Restriction digest of DNA	Developmental stage[a] tissue[b]
1	2 hr	10	180–250	*Bam*H I	Infertile egg/GD
2	2 hr	5	70–150	*Bam*H I	2–16 Cells/E
3	24 hr	13	50–180	*Bam*H I	St. 1/E or GD
4	2 days	5	53–90	*Bam*H I	St. 3–4/E or B
5	3 days	6	53–75	*Bam*H I	St. 10–13/E
6	4 days	8	65–90	*Bam*H I	St. 13–17/E, EB, or B
7	5 days	8	65–75	*Bam*H I	St. 18–24/E, EB
8	6 days	23	17.5–140	*Bam*H I	St. 24–27/E, EB
9	7 days	38	17.5–140	*Bam*H I	St. 27–29/E, EB
10	2 hr	10	45–100	*Nru* I	2–32 Cells/E
11	24 hr	10	36–75	*Nru* I	St. 1/E or GD
12	7 days	6	12–45	*Nru* I	St. 24–29/E, EB
13	2 hr	5	75–90	Uncut monomers	2–8 Cells/E
14	26 hr	5	65–75	Uncut monomers	St. 1/E or GD

[a]Developmental stages according to Hamburger and Hamilton (1951).
[b]GD, germinal disc (indicative of infertility or early failure of development); B, blastodermal tissue (indicative of impaired embryogenesis); E, embryo; EB, extraembryonic blastoderm.

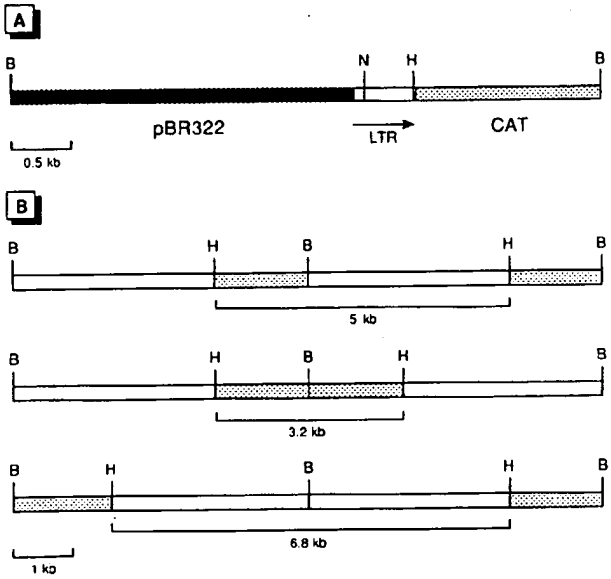

Fig. 2. A: Restriction map of pRSVcat showing restriction sites referred to in text and position of plasmid vector and insert sequences. B, BamH I; N, Nru I; H, Hind III. B: Possible orientations of pRSVcat linear molecules after random ligation. Hind III restriction fragment sizes are indicated.

also analysed after Hind III digestion. The restriction fragment patterns generated, when samples from 2 hr cultures (experiment 2) were probed with pRSVcat, are shown in Figure 3C. Restriction fragments of 6.8, 5, and 3.4 kb were detected. The observed pattern can be generated if the BamH I-linearised pRSVcat molecules are ligated together in random orientation: Head-to-tail ligation will result in 5 kb Hind III fragments, tail-to-tail ligation in 6.8 kb fragments, and head-to-head ligation in 3.4 kb fragments (Fig. 2B). This pattern was seen in all samples in which the injected sequences were detected.

These results indicate that injected DNA molecules ligate rapidly in random orientation to produce large concatemeric molecules of pRSVcat. If DNA from the cultured embryos is digested with BamH I, most of the pRSVcat is reduced to the original 5 kb monomer (see Fig. 5). This demonstrates that the BamH I sites are regenerated by ligation of the complementary ends of the injected linear molecules, which presumably are not degraded before ligation occurs.

Fertilised ova were also injected with pRSVcat linearised with the restriction enzyme Nru I, which generates blunt ends (see Fig. 2A). Samples were cultured for 2 hr, 24 hr, and 7 days (Table 2, experiments 10–12). The DNA extracted was analysed as described above. The results were essentially the same as when BamH I cut DNA was injected (data not shown). Blunt-ended DNA molecules, therefore, also rapidly ligate after injection into the germinal disc to form random concatemeric molecules.

Circular Molecules Recombine After Injection

A preparation of pRSVcat, mainly in monomeric, supercoiled circular form, was injected into fertilised ova, which were cultured for 2 and 26 hr (Table 2, experiments 13 and 14). Representative results of analysis of total DNA extracted from individual cultures, without digestion or after Hind III digestion, are shown in Figure 4. After 2 hr incubation, the plasmid was detected on gels as existing in relaxed circular form, presumably a result of nicking of the supercoiled DNA. The DNA is probably nicked during preparation of total nucleic acid from the embryos. Supercoiled plasmid DNA mixed with control embryo material before nucleic acid extraction is detected as relaxed circular molecules in the extract. After 26 hr, the plasmid was found as high-molecular-weight DNA,

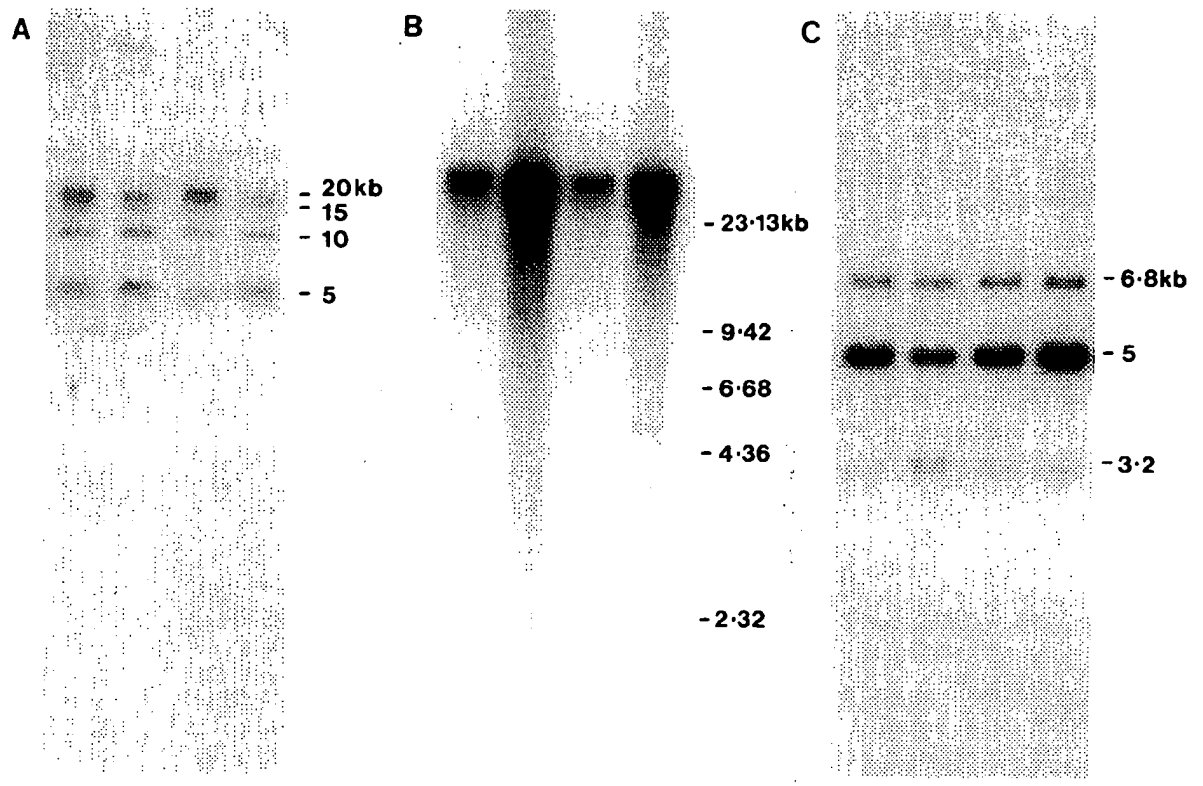

Fig. 3. A: DNA extracted from four infertile eggs after injection with BamH I-linearised pRSVcat into fertilised ova, probed with pUC9. B: DNA extracted from four embryos cultured for 24 hr after injection of BamH I-linearised pRSVcat into fertilised ova, probed with pUC9. C: Hind III restriction digests of DNA extracted from embryos cultured for 2 hr after injection of BamH I-linearised pRSVcat into fertilised ova, probed with pRSVcat.

with a small proportion persisting as monomeric closed circles. Hind III digestion of both the 2 hr and 26 hr samples generated 5 kb linear molecules. This suggests that the high-molecular-weight molecules are generated by recombination between circular molecules, resulting in head-to-tail concatemers.

These observations led to reexamination of the results of injecting linear pRSVcat. The autoradiograms of Hind III digests of DNA from 24 hr cultures, probed with pRSVcat, were analysed by scanning densitometry. The amount of 5 kb fragment present was 2–4.8-fold greater than would be expected were the concatemers produced only by random ligation of linear molecules. Recombination between circular plasmid molecules, either monomers or concatemers, could produce this excess of plasmid copies in head-to-tail orientation.

Injected DNA Is Replicated and Persists During Embryogenesis

The amount of pRSVcat present in cultures at different times after injection was estimated. Two samples were analysed from each time point between 2 hr and 7 days (Table 2, experiments 2–9). A known volume of the total DNA from each sample was digested with BamH I, and the intensity of hybridisation to plasmid vector probe was compared to that of known amounts of pRSVcat run on the same gel. The approximate amount of pRSVcat present in each culture was calculated from this information and compared with the amount of DNA initially injected. After 2 hr incubation, two to three times less pRSVcat was detected than was originally injected, but, after 24 hr, 10–20 times as much pRSVcat was present. This indicates that initially some of the plasmid DNA was lost but the remaining DNA was then replicated, possibly by as much as 50-fold. After 2 days of incubation, the amount of plasmid DNA detected began to decrease, and, by 3–4 days in culture, the amount of pRSVcat present approximately equalled the amount that had been injected. Representative results are shown in Figure 5.

The plasmid was detected in all the embryos injected and cultured for 2 and 24 hr and in 80–90% of the 2, 3, and 4 day cultures. Between days 5 and 7 of incubation, the proportion of cultures with detectable amounts of pRSVcat decreased (Table 3). The level of detection in these experiments is below 5 pg of pRSVcat as deter-

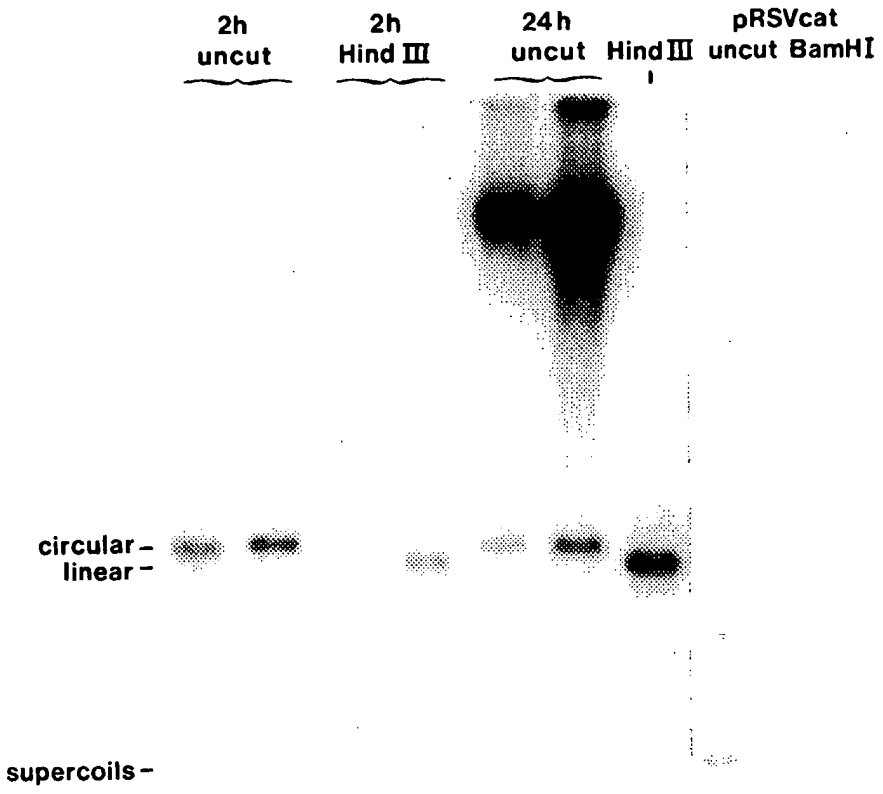

Fig. 4. DNA extracted from embryos injected with mainly supercoiled pRSVcat followed by in vitro culture for 2 and 24 hr. Samples are shown unrestricted and restricted with Hind III and probed with pUC9.

mined by a control of 5 pg of plasmid on each gel. By 7 days, ~30% of the cultures contained detectable amounts of plasmid, most commonly in the extraembryonic blastoderm, less frequently in both embryo and blastoderm, and rarely in the embryo alone.

No Evidence for Integration of Plasmid DNA

Integration of the injected plasmid into the host chromosomes would generate novel restriction fragments from the junctions between the plasmid DNA and chick genomic DNA. Sixty-one embryos injected with BamH I-linearised pRSVcat and cultured for 6 or 7 days were analysed (Table 2, experiments 8 and 9). The autoradiograms of Southern transfer experiments of Hind III digests of total DNA were examined for the presence of hybridising restriction fragments of sizes other than those expected from self-ligation of the injected molecules. The probe used in these experiments was the plasmid vector sequence. Therefore, only the junction between the vector region of pRSVcat (3.2 kb Hind III–BamH I fragment; Fig. 2A) and genomic DNA would have been detected; 5 μg of total DNA from each sample was digested, and the control sample of 5 pg of pRSVcat was visible on all autoradiograms. The sensitivity of these experiments was approximately one copy of pRSVcat in two to four cells. No junction fragments were detected. We conclude from these experiments that there is no evidence for integration of the plasmid DNA into the host genome. If integration occurred after the first or second mitotic divisions, a particular junction fragment would be present in only a proportion of the cells of an embryo and would not have been detected.

Expression of the Reporter Gene

CAT enzyme activity was assayed to determine qualitatively if the sequences present on the injected DNA were expressed. Cultures were assayed 2, 6, and 7 days after injection. Seven embryos cultured for 48 hr, to the primitive-streak stage, were divided in half. One-half of each sample was assayed for CAT activity, and DNA extracted from the remaining material was analysed in Southern transfer experiments as before. The results of this experiment are shown in Figure 6. Plasmid DNA was present in all the samples, and CAT enzyme activity was also detected. Although this analysis was not quantitative, there was no obvious correlation between the amount of plasmid present and the level of enzyme activity.

Samples of extraembryonic blastoderm, retained

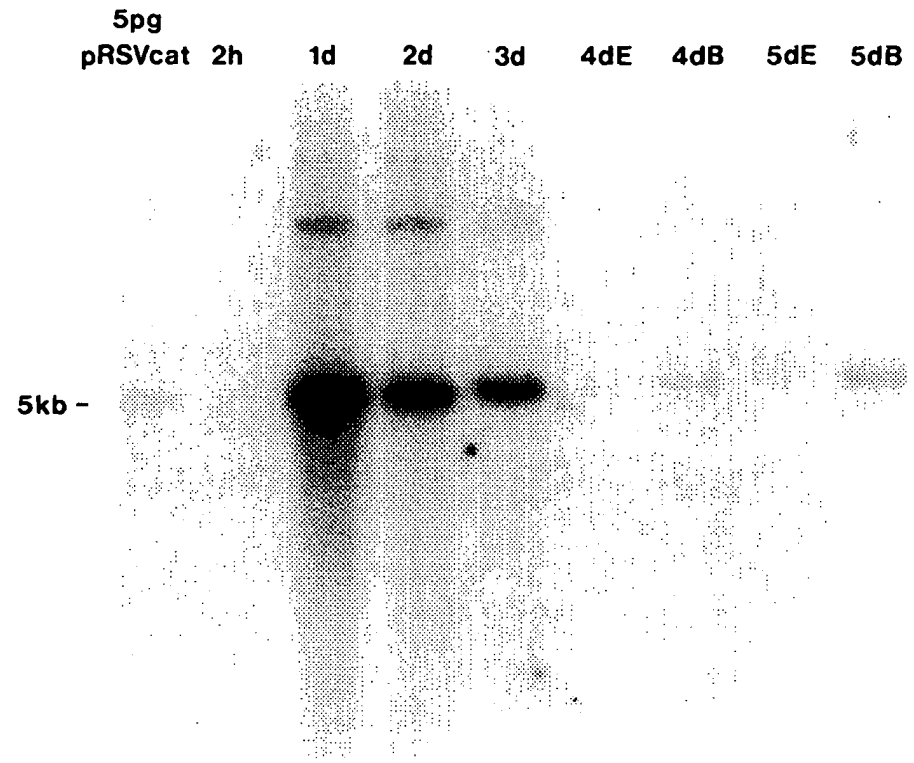

Fig. 5. BamH I restriction digests of DNA extracted from embryos injected with ~50 pg of BamH I-lineared pRSVcat and cultured for 2 hr or 1, 2, 3, 4, or 5 days; 15% of each DNA preparation was digested for each time point. The day 4 and day 5 cultures were separated into embryo (E) and extraembryonic blastoderm (B) samples. The filter was probed with pUC9.

TABLE 3. Detection of Plasmid Sequences in 5, 6, and 7 Day Embryo Cultures

Tissue ± plasmid[a]	Culture period (days) 5d	6d	7d
E^-EB^-	2	20	27
E^+EB^-	1	0	0
E^-EB^+	0	2	7
E^+EB^+	5	1	4
Total	8	23	38

[a]E, embryo; EB, extraembryonic blastoderm; −, no detectable plasmid; +, plasmid sequences detected.

from day 6 and day 7 cultures (Table 2, experiments 8 and 9), were assayed for CAT activity. Sixty-eight samples were analysed, and the enzyme activity was correlated with the presence or absence of detectable pRSVcat DNA; 51 samples had no detectable CAT activity or hybridisation of plasmid sequences, 11 had a low level of CAT activity and of plasmid DNA, three showed no enzyme activity but plasmid sequences were present, and three had a low level of enzyme activity but no detectable plasmid DNA. Although by day 7 in culture both the presence of pRSVcat DNA and the enzyme activity were barely detectable in these experiments, it is clear that the injected DNA could be expressed.

DISCUSSION

We have performed experiments to analyse the fate of cloned DNA injected into the cytoplasm of the chick fertilised ovum before the first cleavage division. After injection, linear DNA molecules ligated rapidly to form large random concatemeric molecules. Recombination involving circular molecules resulted in tandem copies of the injected plasmid in direct orientation. We observed replication of the injected DNA during the first 24 hr of development, a period of rapid cell division leading to formation of a blastoderm comprising some 60,000 cells. Over the subsequent days of incubation, the exogenous DNA was apparently diluted out and lost, although it was still detected in some cultures after 7 days.

Within 24 hr of injection into the germinal disc, plasmid DNA was detected on gels as high-molecular-weight DNA. Incorporation of injected DNA into high-molecular-weight DNA has also been detected in *Xenopus* (Etkin et al., 1984), sea urchin (McMahon et al., 1985), and trout (Chourrout et al., 1986). These results

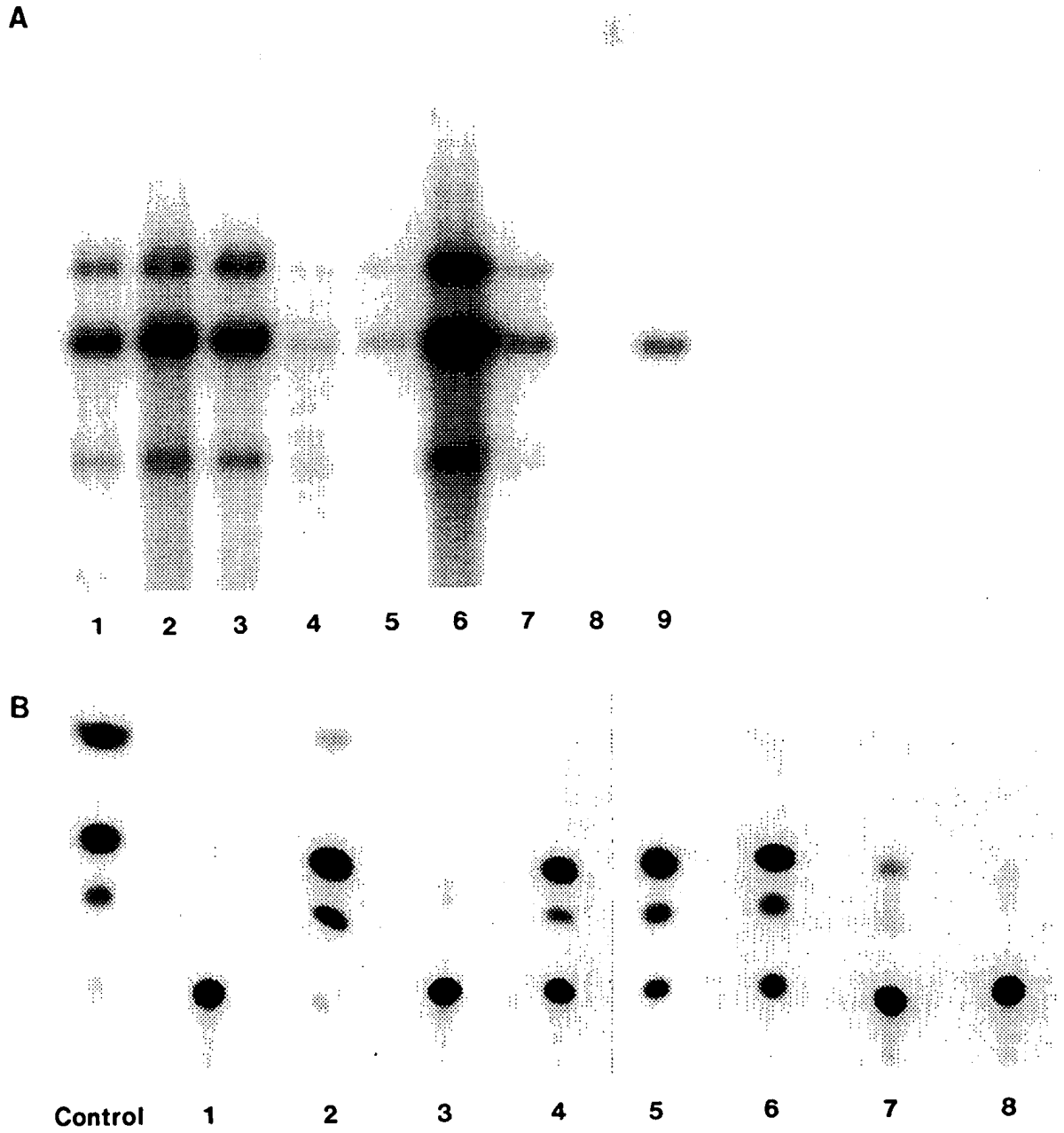

Fig. 6. A: Hind III digests of DNA prepared from half embryo samples injected with BamH I-linearised pRSVcat and cultured for 2 days (1–7), uninjected control sample (8), and 5 pg pRSVcat control (9), probed with pRSVcat. B: CAT enzyme assays of the remaining samples from A, samples 1-8. Control sample 5 units of CAT (Pharmacia).

have been interpreted as indicating incorporation of the plasmid into genomic DNA, but further evidence is required to support this suggestion. Chromosomal integration of foreign DNA can be demonstrated by the presence of novel restriction fragments representing the junctions between the foreign DNA and genomic DNA. Novel restriction fragments could be generated by rearrangements within plasmid concatemers. Isolation of such restriction fragments and demonstration of the juxtaposition of foreign DNA and genomic se-

quences is conclusive evidence for integration. We have not detected any putative junction fragments in the relatively small number of samples analysed. They may also have been below the level of detection in the experiments described. Although we have yet to demonstrate chromosomal integration of DNA injected into the cytoplasm of chick fertilised ova, we have shown that such manipulations are possible. We intend to continue looking for evidence for integration using short-term cultures and to follow the fate of injected DNA in embryos cultured for longer periods through to hatch. Application of the method described to the efficient production of transgenic poultry may involve the use of a vector system.

We detected expression of the exogenous DNA by assaying CAT gene activity in day 2 and day 7 cultures. The Rous sarcoma virus LTR, which directs expression of the CAT gene, has high activity in chick embryo fibroblasts (Gorman et al., 1982a), although RSV itself is not expressed when integrated in chick early embryonic cells (Mitrani et al., 1987). Tissue-specific gene expression during early development, from injected sequences that are episomal, has been shown in *Xenopus* (Wilson et al., 1986; Mohun et al., 1986) and sea urchin (Flytzanis et al., 1987). It may be possible to use the method described here to analyse gene expression in early development in the chick by looking for regulation of expression of particular cloned genes after injection of suitable constructs.

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Original Article

Embryonic expression of ß-actin-lacZ hybrid gene injected into the fertilized ovum of the domestic fowl

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ABSTRACT An experiment was carried out to investigate the expression of cloned DNA injected into the germinal disc of the chick fertilized ovum. The ß-actin-lacZ hybrid gene, MiwZ, was injected, in the closed circular form, into the cytoplasm of the germinal disc at the single-cell stage. The embryos were cultured *in vitro*, then in recipient eggshells up to day 4 of incubation. The survival rate of the embryos at day 4 was 42% (55/130), and the rate of embryos expressing MiwZ was 64% (35/55). Twenty-two embryos expressed the MiwZ in both embryonic and extraembryonic tissues, while the remainder expressed the MiwZ in only extraembryonic tissues. Mosaic expression was observed in most of the embryos expressing MiwZ in embryonic tissues. Expression throughout all tissues of the embryo including blood cells occurred in one case. In this case, the injected DNA was assumed to have integrated at an earlier stage. The results indicate that it is now possible to investigate the promoter activities of introduced exogenous genes as well as the effect of introduced genes on embryogenesis in early chick embryos. This technique may also facilitate the production of transgenic chicks.

KEY WORDS: *chick, germinal disc, microinjection, lacZ*

Introduction

Perry (1988) developed a complete culture system for the chick embryo from a single cell to hatching. Recently, Naito *et al.* (1990) have improved this method and obtained a much higher rate of hatching (34.4%) of fertilized ova grown in culture. If cloned genes can be introduced into single-cell stage embryos and the recipients are developed *in vitro*, this will prove a powerful method for analyzing embryogenesis at the molecular level.

Various methods of gene transfer to chicks have been employed in attempts to obtain transgenic chicks. Transgenic chicks have been produced successfully using retroviruses as a vector (Souza *et al.*, 1984; Shuman and Shoffner, 1986; Salter *et al.*, 1986, 1987; Hippenmeyer *et al.*, 1988; Bosselman *et al.*, 1989; Crittenden *et al.*, 1989; Salter and Crittenden, 1989). This method, however, has disadvantages such as a limitation in size of the inserted genes and difficulties in preparation of high-efficiency infectious viruses. Petitte *et al.* (1990) have succeeded in developing a technique for blastodermal cell transfer and attempted to produce transgenic chicks from blastodermal chimeras (Gibbins *et al.*, 1990). Sang and Perry (1989) microinjected the bacterial CAT gene into the fertilized ovum at the single-cell stage and analyzed the fate of the injected DNA during development. They reported that some of the DNA was lost after injection, but that the remainder was replicated approximately 20-fold during the first 24 h of development. Between day 1 and day 7 in culture, the DNA was gradually lost and diluted out as the embryos developed. No evidence for chromosomal integration of the exogenous DNA was obtained, suggesting that the plasmid DNA persisted episomally. These observations suggest that the microinjection method may not be appropriate for the efficient production of transgenic chicks, but could be suitable for analyzing the expression and the effect of microinjected genes during early development of chick embryos. Injected DNA may be expressed extrachromosomally without position effect during the dynamic morphogenetic events occurring in the first 3 days of chick development. To examine whether microinjected DNAs are efficiently expressed in early embryos, we introduced the *E. coli* ß-galactosidase gene recombined with the chicken ß-actin promoter/enhancer and the RSV enhancer element into fertilized eggs, and obtained the expression of the exogenous gene at high frequency.

Abbreviations used in this paper: RSV, raus sarcoma virus; LTR, long terminal repeat; CAT, chloramphenicol acetyltransferase; ES cell, embryonic stem cell.

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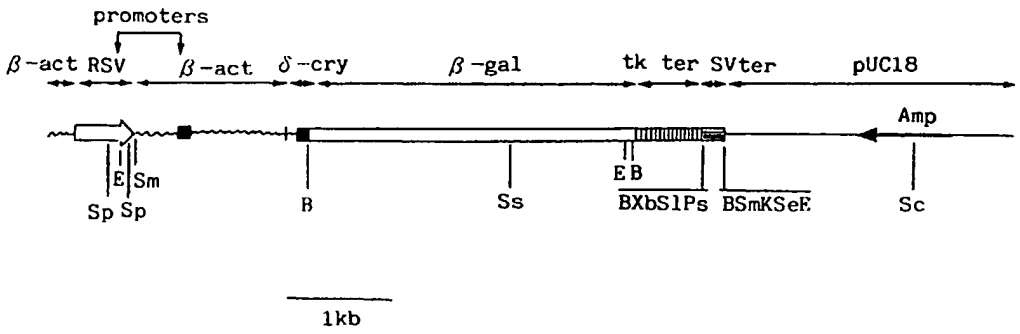

Fig. 1. Structure of the recombinant plasmid, MiwZ. *ß-actin (ß-act) and δ-crystallin (δ-cry) sequences are shown by wavy lines and their exon sequences by the solid boxes. Bacterial sequences (ß-gal) are indicated by open box, HSV-tk sequences by box with vertical stripes, SV40 sequences by box with horizontal stripes, the RSV LTR sequences by the open arrow, and plasmid vector sequence by the horizontal line. Promoters and termination signals (tk ter and SV ter) are also indicated. Restriction sites: B, BamHI; E, EcoRI; K, KpnI; Ps, PstI; Sc, ScaI; Sl, SalI, Sm, SmaI; Sp, SphI; Ss, SstI; Xb, XbaI.*

Results

Introduction of the MiwZ by oblique or vertical injection

The pronuclei in the central region of the germinal disc are masked by yolk spheres and could not be identified by microscopy. For the preliminary experiments, the micropipette was inserted obliquely into the central site of the germinal disc and DNA was injected into the cytoplasm of fertilized ova, followed by up to 4 days of embryo culture. Expression of the MiwZ was detected in most cultures, but the area of the expression of MiwZ was spread in a radial manner from the injection site at the blastoderm stage (Fig. 2A). In 3-day-old embryos, expression of the MiwZ was observed frequently in the extraembryonic tissues in a radial manner as in Fig. 2B, but no embryonic tissue expression was observed in any of the cultures. These results suggest that injected DNAs do not diffuse through the cytoplasm of fertilized ova, and that only the cells derived from the injected region incorporate exogenous DNA into the nuclei and express MiwZ. Thus, in the next experiments, the micropipette was inserted vertically into the center of the germinal disc, in order to inject DNAs into that part of the cytoplasm which contributes to embryonic tissues and germ cells (Ginsburg and Eyal-Giladi, 1987). As expected, embryonic tissue expression of the MiwZ was observed in some cultures by vertical injection.

DNA concentration, embryonic development and MiwZ expression

The results of the expression of MiwZ injected vertically into the germinal disc are shown in Table 1. When the DNA concentration was 1.0 mg/ml, only 1 out of 62 manipulated embryos survived on day 4 of incubation. Development of most embryos was arrested at the early blastoderm stage. High doses of DNA may be very toxic for chick embryos, as found for *Xenopus* embryos (Gurdon, 1974). On the other hand, when the DNA concentration was 0.1 mg/ml, the survival rate of the manipulated embryos was 42% (55/130) on day 4 of incubation. In 64% (35/55) of them the MiwZ was expressed in the embryonic or extraembryonic tissues. Embryonic expression of the MiwZ was detected in 22 (40.0%) out of the 55 embryos surviving on day 4 of incubation, and in the remaining 13 embryos (23.6%) the MiwZ expressed in the extraembryonic tissues only.

MiwZ expression in the embryonic tissues

Fig. 4A shows an embryo cultured for 4 days after injection of water. The embryo developed normally, and no endogenous ß-galactosidase activity was detected at this stage by our histochemical procedure. Embryos shown in Figs. 3A, 3B and 3C, cultured for 3 or 4 days, show expression of the MiwZ in the embryonic as well as extraembryonic tissues, but the expression pattern is mosaic. Fig. 3A illustrates a 3-day embryo showing expression of the MiwZ in the embryonic and extraembryonic tissues. The MiwZ is expressed almost throughout the embryos, but in a mosaic manner. A radial staining pattern in each somite and a columnar staining pattern in the neural tube are observed (see insert). Figs. 3B and 3C illustrate other embryos incubated for 4 days. In the embryo shown in Fig. 3B, a columnar staining pattern of the neural tube is clear. In Fig. 3C, the expression of MiwZ can be mainly recognized in the heart and neural tube. Table 2 shows the site of expression of MiwZ in the 22 embryos incubated for 4 days. The incidence of expression was higher in the neural tube (64%) than in other organs and tissues. Fig. 4B shows an intensely stained embryo incubated for 4 days. This was the only case in which the expression of MiwZ occurred throughout the embryo, probably in all cell types including some blood cells.

Discussion

In this study, we succeeded in obtaining expression of the MiwZ in both embryonic and extraembryonic tissues by injecting the

TABLE 1

EXPRESSION OF MiwZ IN CHICK EMBRYOS AT 4 DAYS FOLLOWING INJECTION INTO THE CHICK GERMINAL DISC

DNA concentration	Number of eggs manipulated	Number (%) of embryos on day 4	Number of embryos (%) showing expression of MiwZ			
			No expression	Extraembryonic tissues only	Part* of embryo	Whole* embryo
1 mg/ml	62	1 (1.6)	0 (0.0)	1 (100.0)	0 (0.0)	0 (0.0)
0.1 mg/ml	130	55 (42.3)	20 (36.4)	13 (23.6)	21 (38.2)	1 (1.8)

*Embryonic and extraembryonic tissue expression

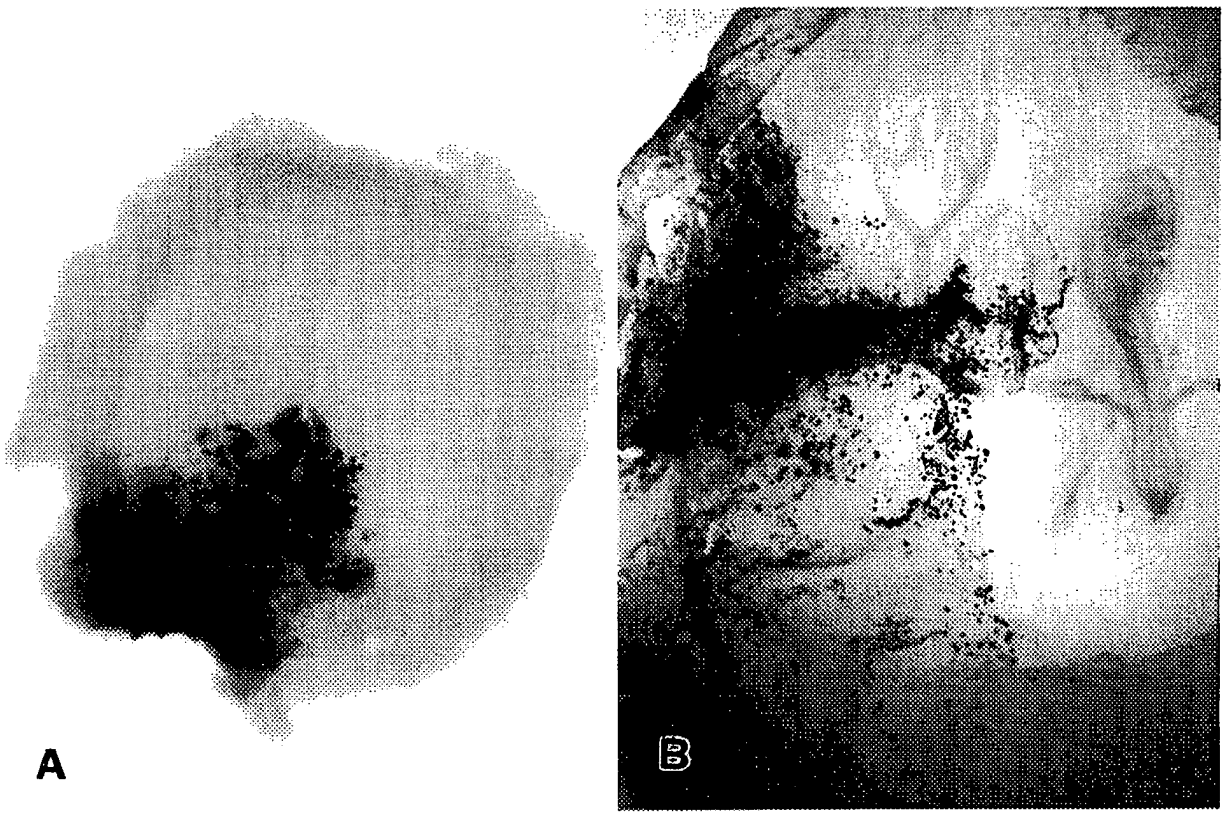

Fig. 2. Expression of the MiwZ by oblique injection. *The MiwZ was injected obliquely into the germinal disc of the fertilized ovum at the single-cell stage. Expression of the MiwZ was analyzed after 1 day* **(A)** *and 3 days* **(B)** *of incubation.*

corresponding DNA into the cytoplasm of the chick germinal disc at the single-cell stage. This technique enables us to investigate the promoter activities of introduced genes and also the effect of introduced genes, the products of which are expected to regulate chick embryogenesis at the molecular level. In this study, we obtained one promising case in which the MiwZ was expressed throughout the embryo (Fig. 4B). In this case the injected DNA might have been incorporated into the nucleus at the single-cell stage, and the DNA integrated in the host chromosome at an early stage of development. This suggests that it may be possible to produce transgenic chicks by the method of injecting DNA into the cytoplasm of the germinal disc at the single-cell stage. Most of the embryos expressing the MiwZ in this study were mosaic. This kind of mosaicism is also observed in *Drosophila* and *Xenopus* when DNA is microinjected into the cytoplasm (Steller and Pirrotta, 1985; Etkin and Pearman, 1987). The oblique injection experiment indicated that injected DNA does not diffuse throughout the cytoplasm of the germinal disc (Figs. 2A-B). If DNA is injected at a site far from the first cleavage plane, it will not be distributed in all of the cells of the embryo. In such a case DNA might be introduced only into one of the two daughter cell nuclei after the first cleavage or a small population of cells at a later stage, so that the distribution of DNA would become mosaic in the embryos or extraembryonic tissues. In order to minimize this kind of mosaicism, the micropipette was inserted «vertically» into the central site of the germinal disc close to the pronuclei. Since the cytoplasm of the chick germinal disc is opaque and the thickness of the germinal layer is about 100 μm (Kochav *et al.*, 1980), it is difficult to position the tip of the micropipette in the germinal layer. Sang and Perry (1989) marked the micropipette at a distance of 150-200 μm and 2.0-2.5 mm from the tip to determine the depth of penetration into the cytoplasm and the volume of DNA, respectively. By their method, it seems difficult to inject DNA into the germinal layer close to the upper surface. It may be important to inject DNA into the central part of the germinal layer close to the upper surface because embryonic tissues and germ cells may originate from the central zone of the area pellucida (Ginsburg and Eyal-Giladi, 1987). In order to overcome the difficulty of injecting DNA into the germinal layer, a continuous flow of solution from the micropipette was maintained while the micropipette was inserted vertically into the central area of the germinal disc. This method ensured that some of the DNA was placed in the cytoplasm of the central area of the germinal layer close to the upper surface. Although the volume of DNA flowing out from the micropipette was not constant, injection into the germinal layer in many of the manipulated eggs appeared to be successful, because the expression of MiwZ was detected in about 60% of the cultures surviving on day 4 of incubation.

In the present experiment, we injected the closed circular form of DNA in order to investigate the transient expression of the DNA, because circular form DNA seems to resist digestion with DNase in

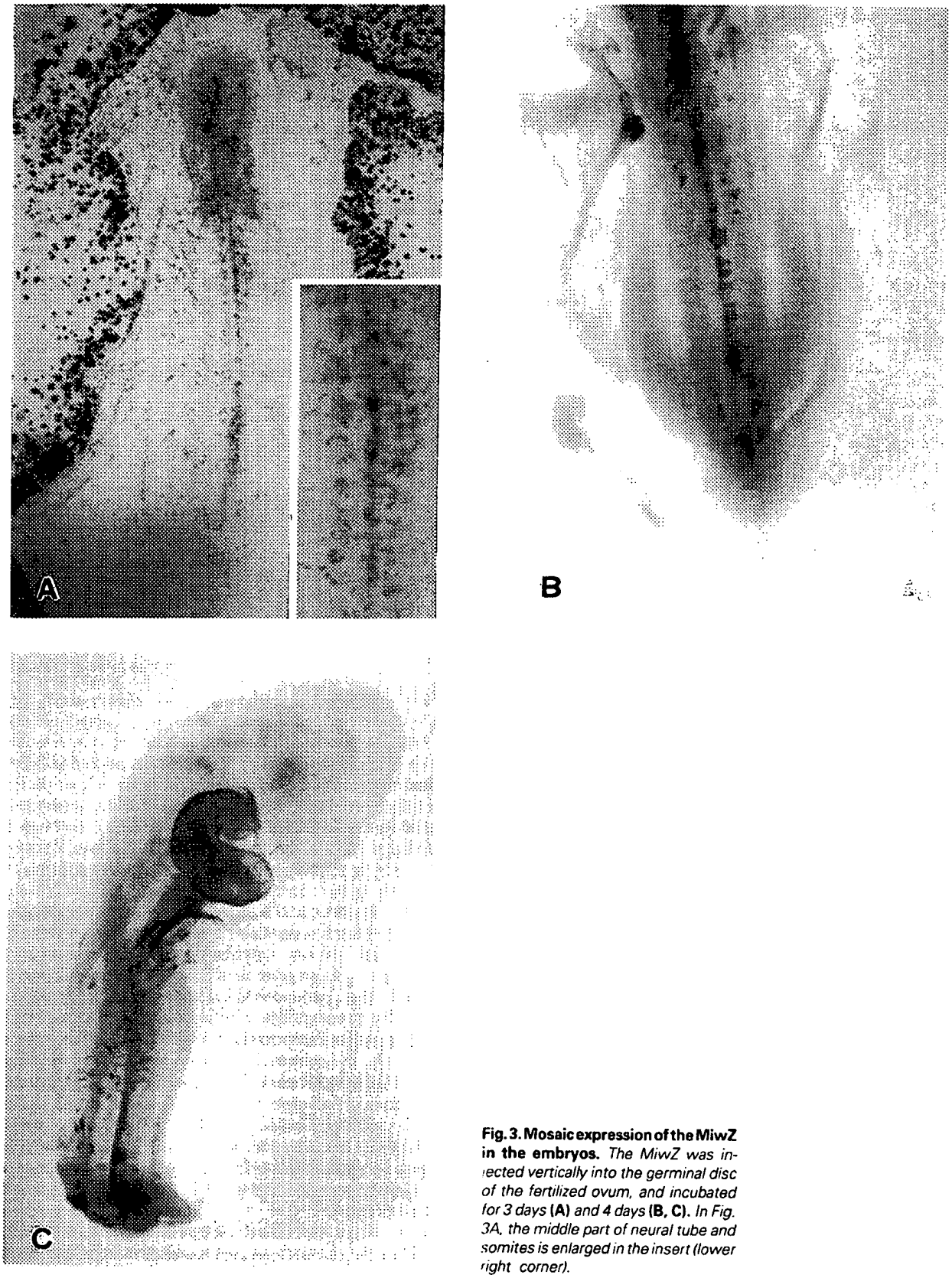

Fig. 3. Mosaic expression of the MiwZ in the embryos. *The MiwZ was injected vertically into the germinal disc of the fertilized ovum, and incubated for 3 days* **(A)** *and 4 days* **(B, C).** *In Fig. 3A, the middle part of neural tube and somites is enlarged in the insert (lower right corner).*

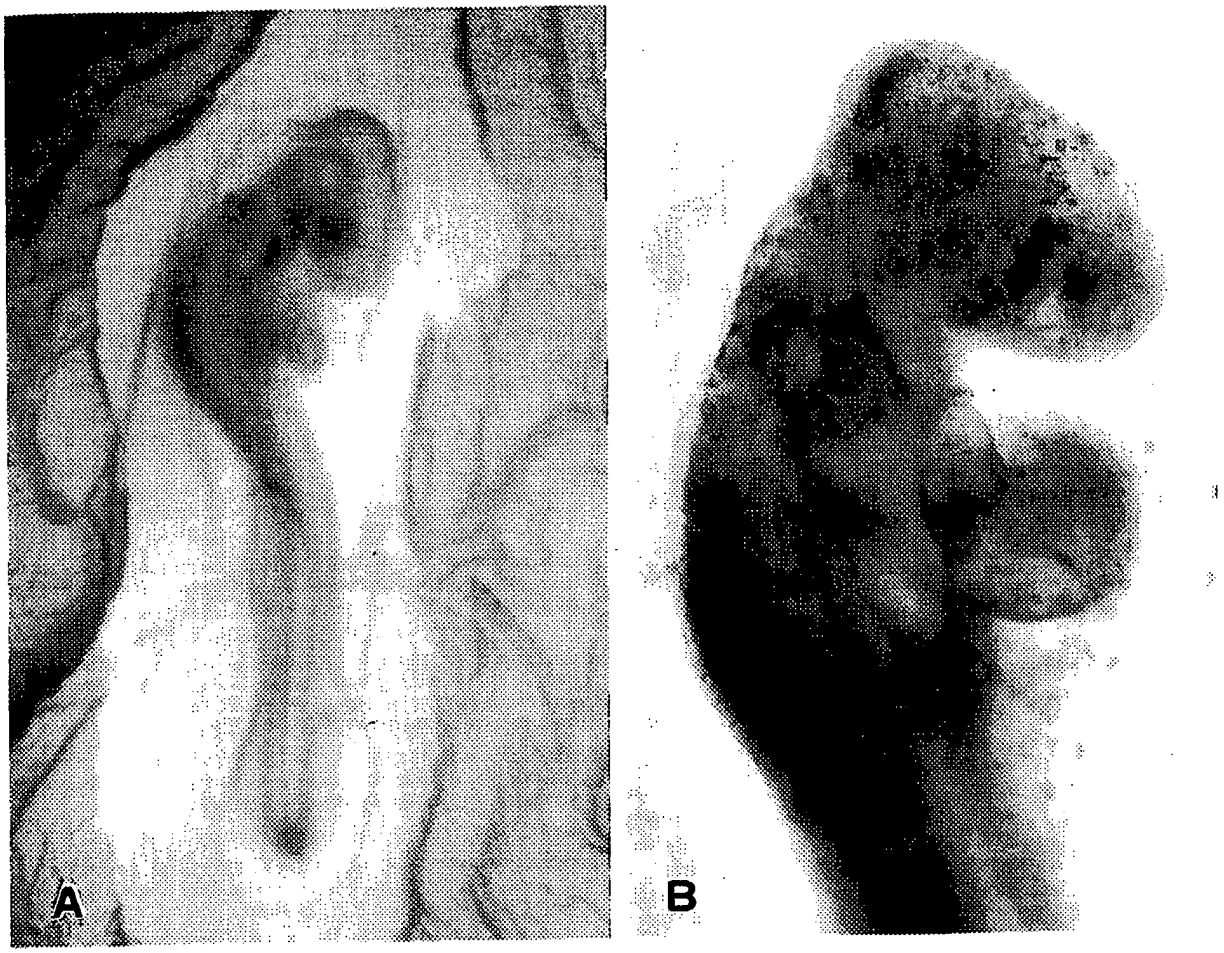

Fig. 4. Expression of the MiwZ throughout the embryos. *The MiwZ was injected into the germinal disc of the fertilized ovum and incubated for 4 days* **(B)**. *A control embryo was incubated for 4 days and stained* **(A)**.

the cytoplasm (Fu *et al.*, 1989) and to be maintained extrachromosomally to a later stage. Sang and Perry (1989) reported that the linear DNA molecules injected into the germinal disc are ligated rapidly after injection to form random concatamers. However, they did not obtain any evidence that injected DNA is incorporated into the host chromosomes from analysis of the fate of injected DNA in 253 embryos. In the present experiment, we have only analyzed expression of the injected DNA but not its fate. However, the injected DNA is assumed to have been integrated at an earlier stage in at least one case (Fig. 4B). This suggests that some closed circular DNA molecules could be incorporated into the host chromosomes, and further analysis of the fate of DNA injected by our method is planned.

The transcriptional activity of the MiwZ construct is very strong. Most positive cells are detected within 5-10 min of incubation in the X-gal solution. We have also obtained intense staining in all types of cultured cells of chick embryos transfected with MiwZ (data not shown). In mice, Suemori *et al.* (1990) obtained ES cells, in which MiwZ is uniformly expressed. All derivatives of these ES cells in chimeras expressed MiwZ. These data suggest that all cells incorporating MiwZ can express MiwZ, and that the mosaic expression may result from the non-uniform distribution of MiwZ, but not from differential on the promoter activity. However, in the present experiment the MiwZ expression tended to be exhibited by the extraembryonic membranes and neural tube (Table 2). It is supposed that this tendency may reflect the proliferation rate of cells, because DNA existing extrachromosomally is diluted out by proliferation.

Materials and Methods

Preparation of the fertilized ova

White Leghorn hens were artificially inseminated. The hens were killed by intravenous injection of sodium pentabarbitone (100-150mg/bird) at 2.75 h after oviposition of the preceding egg, and fertilized ova were obtained from the magnum of the oviduct. At this time the fertilized ovum is at the precleavage stage and the pronuclei are swelling. Syngamy takes place within 1 h (Perry, 1987). Ova were placed in glass jars of adequate size which contained approximately 5 ml of culture medium consisting of thin albumen and salt solution (3:2) according to the method of Perry (1988).

TABLE 2

SITE OF EXPRESSION OF MiwZ IN 4 DAY INCUBATED CHICK EMBRYOS

Expression site	Number of embryos*	Percentage
Neural tube	14	64
Ectoderm	8	37
Heart	5	23
Blood cell	5	23
Whole embryo	1	5

*Number out of 22 embryos that expressed the MiwZ

The gene to be introduced

The structure of the constructed plasmid, MiwZ, used in this experiment was previously described by Suemori *et al.* (1990) (Fig. 1). It contains *E. coli* ß-galactosidase (lacZ) gene under the control of RSV enhancer and chicken ß-actin gene promoter/enhancer. The circular form plasmid was suspended in distilled water and the concentration of DNA was 1.0 mg/ml or 0.1 mg/ml.

Microinjection of DNA

Micropipettes were made by pulling 1.0 mm siliconized microcapillary tubing. The tips were beveled down (20-25°) to an outside diameter of 10-15 µm. The micropipette was held by a micromanipulator (M-152, Narishige, Tokyo) and connected to a microinjector (IM-5B, Narishige, Tokyo) with a teflon tube (inside diameter 1 mm, outside diameter 2 mm). A pressure-lock gas syringe was connected to the mid point of the tube so that the pressure in the micropipette could be monitored. The tube was filled with distilled water and the micropipette containing DNA was filled with liquid paraffin in order to remove the air from the tube and the micropipette completely.

The micropipette was inserted into the central area of the germinal disc up to a depth of about 150 µm vertically or obliquely (30° from the vertical) through the albumen capsule and vitelline membrane. A continuous flow system was employed for DNA injection. The injection volume of the DNA was 10-50 nl.

Culture of eggs

The injected eggs were incubated individually for 24 h at 41.5°C in the glass jars sealed with plastic film. The embryos (yolks) were then transferred to recipient eggshells after removing the thick albumen and culture medium (Naito *et al.*, 1990). The shells were filled with thin albumen, and sealed with cling film secured by plastic rings and elastic bands (Perry, 1988). The reconstituted eggs were incubated for 3 days at 38°C and 60% relative humidity, with rocking through an angle of 90°. Embryos were fixed after 1, 3 and 4 days of incubation, and the expression of the injected DNA was analyzed. All procedures for DNA injection and embryo culture were carried out under sterile conditions.

Detection of gene expression

The expression of MiwZ was detected by a histochemical staining method. The embryos and extraembryonic tissues were removed from the yolk in phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS(-)), and fixed with 1% glutaraldehyde in PBS(-) for 15-30 min. After washing three times with PBS(-), the samples were incubated for 2 h at 37°C with 0.05% 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal, Wako, Tokyo), 1mM-$MgCl_2$, 0.1% Triton X, 3mM-potassium ferrocyanide and 3mM-potassium ferricyanide in PBS(-).

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From Cleavage to Primitive Streak Formation: A Complementary Normal Table and a New Look at the First Stages of the Development of the Chick

I. General Morphology

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A manual method has been developed to extract eggs from the genital tract without harming the hen. Fourteen developmental stages preceding Hamburger and Hamilton's stage 2 have been studied from live material and photographed from both upper and lower surfaces. Three developmental periods have been distinguished: cleavage, formation of area pellucida, and hypoblast formation. The last two are morphogenetic periods which follow the determination of the embryonic axis. During cleavage the diameter of the germ becomes reduced and the cytoplasmic mass becomes thicker. Later there is a progressive increase of the germ's area. The formation of the area pellucida is a result of an oriented massive loss of the yolk laden cells of the lower layers. The formation of the primary hypoblast is a result of posterio-anteriorly oriented polyinvagination and a later coalescence of the cell aggregates in the same direction. It is not yet possible to conclude whether there is also an anteriorly directed cell movement. The inconsistent usage of the terms blastodisc and blastoderm is discussed. The authors suggest avoiding the term blastodisc and using the general term germ for all the early stages. The term blastoderm may be applied from stage VI onward.

INTRODUCTION

The study of the embryonic development of the chick has been limited for many years almost entirely to the stages from the unincubated blastoderm onward. The reasons for this have been the difficulty of obtaining younger material, for which it was believed that hens had to be sacrificed (Patterson, 1910) and the lack of appreciation of the importance of the earlier stages in embryonic development.

Furthermore, most of the experimental work with chick blastoderms has been performed on embryos from the primitive streak stage and older, on the assumption that the morphogenetic period started with the formation of the streak. This latter assumption was based on the homology which was drawn between the primitive streak and the amphibian blastopore. Development prior to gastrulation in amphibians (or any holoblastic egg) was regarded merely as cleavage, during which period it was generally accepted that "the constituent parts of the cytoplasm of the egg are not displaced to any great extent and remain on the whole in the same positions as in the egg at the beginning of cleavage" (Balinsky, 1970). Thus, before P.S. formation, the chick embryo has been regarded as being in the cleavage period and in possession of no significant morphogenetic activity. Hamburger and Hamilton (1951) have obviously prepared their normal table of the chick to answer to the common needs of most investigators of chick development. It is probably therefore that their stage 1, called prestreak or an unincubated blastoderm, is represented as a disk with no specific details. Their stage 2 blastoderm already contains an initial primitive streak.

The above approach overlooked many known facts concerning the early develop-

[1] Killed in action, October 16, 1973.

ment of the chick embryo. There has been work done on the fertilization and early cleavage in the pigeon (Harper, 1904; Blount, 1909; Patterson, 1909) and in the hen (Goette, 1874; Duval, 1884; Kionka, 1894; Patterson, 1910; Olsen, 1942; Bekhtina, 1960; Emanuelsson, 1965). Some additional work has been done on the formation of the area pellucida (Clavert, 1962) and on the formation of the two-layered blastoderm (Oellacher, 1869; Balfour, 1873; Kolliker, 1875; Koller, 1882; Merbach, 1935; Jacobson, 1938; Peter, 1938; Pasteels, 1945; Spratt and Haas, 1961, 1965; Spratt, 1960; Vakaet, 1962.

However, no coherent picture has been formed to cover the sequence of developmental events from the first cleavage up to primitive streak. The aim of the present study is to fill this gap by presenting a series of normal stages with clear morphologic criteria serving as easy reference points for any investigation on the early stages.

MATERIALS AND METHODS

A technique has been devised to extract eggs mechanically from the hen's genital tract. Hens, either Leghorn or hybrid New Hampshire × Leghorn, were kept in individual cages and artificially inseminated. Prior to a planned extraction of eggs the hens were observed carefully for 1-2 days, and the exact time of laying was recorded for each hen. The time for the first cleavage has been predicted as 6 hr (Patterson, 1910) and 5½ hr (Olsen, 1942) after the laying of the previous egg. Both of these authors report that cleavage begins in the oviduct, but Olsen mentions that it was possible by injecting posterior pituitary extract to obtain eggs 5 hr after the estimated time of ovulation, i.e., 5½ hr after the laying of the previous egg. Olsen and Byerly (1932) describe a method of expelling eggs manually from the hen's uterus, probably at a much later stage. This prompted us to try manual extraction at different times, starting 5½ hr after the laying of the previous egg.

Extraction of the egg was done as follows: First, the hen was checked for the presence of an egg by inserting a finger into the cloaca. Beginning 5½ hr after the laying of the previous egg, the new egg could be palpated in a deep position, probably at the lowest part of the isthmus on transition to the uterus. An egg that could be palpated usually could be expelled manually. The hen was placed on its right side with its left leg held by the left hand of the operator. External pressure was applied with the right thumb to the abdomen anterior to the egg in order to shift it caudally. A second person held the hen by its right leg and caught the egg as it was expelled. This method enabled us to obtain hundreds of eggs at all the desired developmental stages without sacrificing the mothers. The youngest eggs had only a shell membrane, while older ones showed progressive formation of a calcareous shell, the thickness of which was proportional to the duration of its stay in the uterus. The uterine eggs, however, form only part of the material covered in this study; the later stages included in the study are normally laid, unincubated eggs and eggs incubated for various short periods at 37°C. Special attention has been paid to the unincubated eggs, which were collected at the moment of laying and either opened immediately or rapidly cooled and kept at 15°C before being examined.

The eggs were opened into a bowl containing Ringer's solution, the future posterior side of the germ was marked with carbon according to Von Baer's law, and the germ was then dissected out, thoroughly cleaned from adherent yolk, freed of the vitelline membrane, and photographed with a camera mounted on a dissecting microscope. Generally fixation was not employed, but at later stages of development (stage X and onward) a few drops

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of formalin were sometimes added to prevent the thinned-out blastoderms from curling. Each germ was photographed both from its upper and lower sides against a black background.

RESULTS

Hundreds of eggs collected according to the above system included all developmental stages. The stages were defined according to distinct morphological criteria and not to hours of development. The time intervals between the stages are not constant and the approximate uterine age is referred to for each stage. The uterine age was calculated as the interval between the laying of the former egg and the extraction of the studied egg minus 5½ hr. Fourteen stages were defined and described; they encompass three distinct developmental periods: A, cleavage period; B, formation of the area pellucida-period of symmetrization; C, period of hypoblast formation. Roman letters have been used in order to distinguish them from Hamburger-Hamilton.

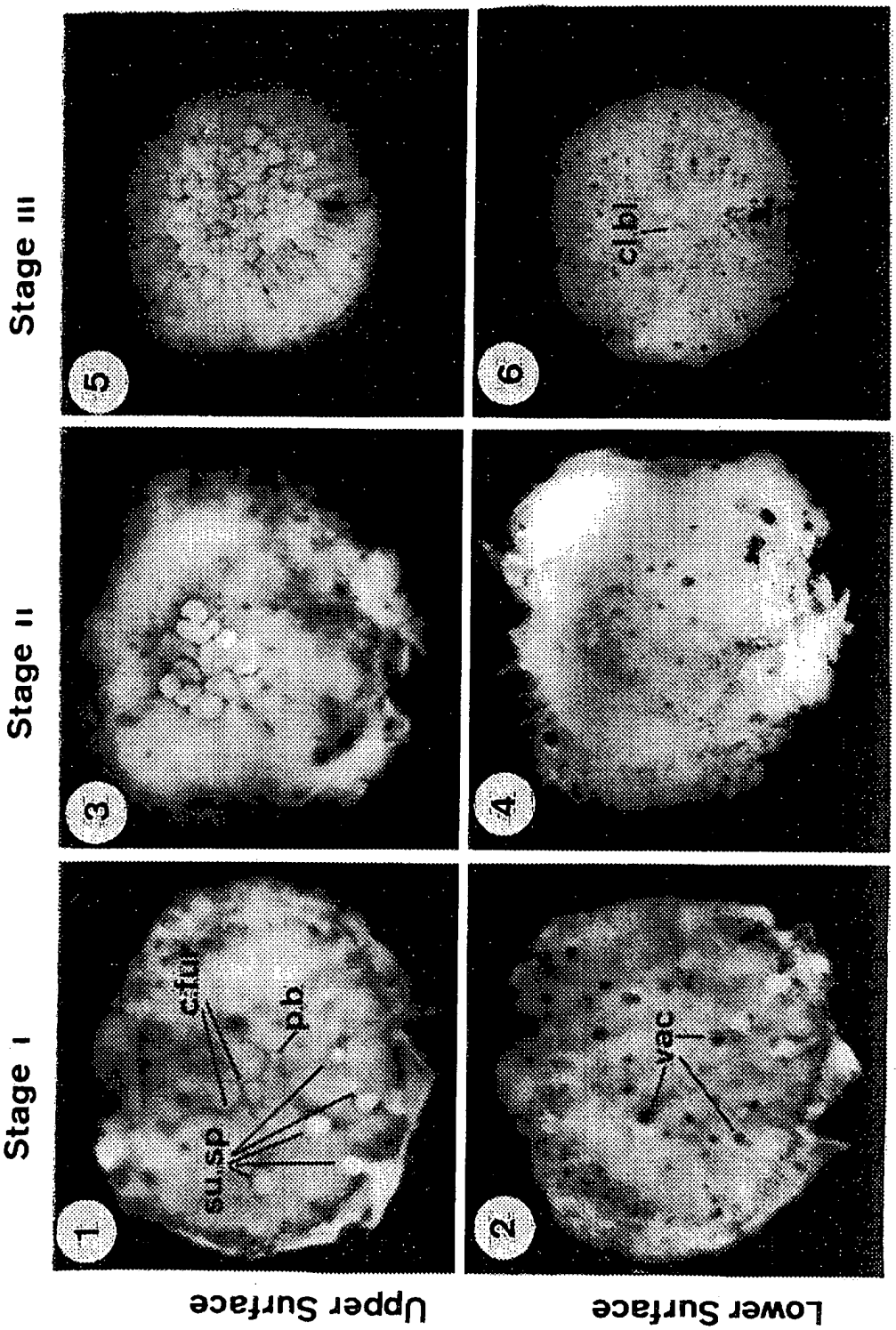
Period A: Cleavage (Stages I-VI)

During this period, the cytoplasmic mass of the germinal disc is cleaving very rapidly, the time span between stages I and VI being about 10-11 hr.

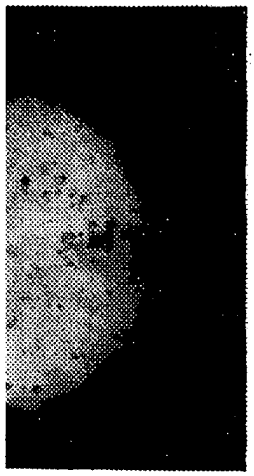
Stage I (0-1 hr uterine age; Figs. 1 and 2). Eggs containing embryos of this stage were extracted from the hens 5½-6½ hr after the former egg had been laid. They were surrounded by a very flaccid shell membrane; the albumen inside the membrane which had not yet absorbed uterine fluid was very viscous. The germinal disc was large (3.5-4 mm in diameter) and in its central region cleavage furrows were seen, sometimes in an excentric position (figs. 31 and 33). As the rate of divisions was very high, five to six divisions during the first 2 hr (from the time of the first cleavage furrow to stage II), we do not refer to each division as a separate stage. We therefore included in stage I the whole range of cleaving patterns in which all cells were still open peripherally, or even those with one to two centrally located and laterally closed cells. Additional stage I germinal discs are shown to demonstrate the variability of cleavage patterns from a very orderly one (Figs. 35 and 36) to another (Figs. 32 and 34) with a single circumscribed cell, indicating asynchrony of the mitotic divisions. The cytoplasm at this stage contains many large vacuoles (vac.). In some of the embryos of this stage a polar body (p.b.) could still be discerned (Figs. 1 and 31). Another relatively common feature is the presence of several knobs (su. sp.) near the periphery, some being minute (the size of a polar body) and others are much larger (the size of a blastomere). The minute knobs resemble the supernumerary sperms of the pigeon (Patterson, 1910), while the larger ones might be nests formed by dividing supernumerary sperms before their disintegration. In a stage I germ observed from the lower side, all the cells seem to be open from below.

Stage II (about 2 hr uterine age; Figs. 3 and 4). On the upper surface there is a group of 14-16 laterally closed cells enclosed by vertical cleavage furrows. The blastomeres are elevated in relation to the originally flat surface of the germ and resemble a nest of eggs. From the central group, cleavage furrows spread out in all directions. The uncleaved cytoplasm still contains vacuoles; however, these are smaller than stage I vacuoles. The lower surface does not yet reveal the formation of horizontal cleavage furrows.

Stage III (3-4 hr uterine age; Figs. 5 and 6). The germ at this stage has a notably smaller diameter than in stage I. This accentuates the tendency, already visible in many stage II germs, that during cleavage the cytoplasmic disc shrinks horizontally and thickens vertically. On the upper surface a central group of 80-90 laterally



FIGS. 1–6.



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FIGS. 1–6.

closed blastomeres is seen. From its margin cleavage furrows radiate to the edge of the germinal disc.

On the lower surface, some 10–16 cells (cl. bl.) that are closed ventrally by horizontal furrows can be seen. The more laterally situated vertical furrows of the dorsal side are not deep enough to show on the vertical surface. In the uncleaved cytoplasmic region of the ventral side many vacuoles can still be seen, although they are much smaller than those of earlier stages.

Stage IV (5 hr uterine age; Figs. 7 and 8). The diameter of the germ is similar to that at stage III. On the upper surface there are 250–300 closed cells while in the center of the lower surface 80–90 ventrally closed cells may be seen. The ventral area covered by the closed cells indicates the dimensions of the subgerminal cavity (s.c.) at this stage.

Stage V (8–9 hr uterine age; Figs. 9 and 10). Cleavage is much more advanced. The closed blastomeres occupy equally large areas both on the upper and lower surfaces. The sub-blastodermic cavity has increased remarkably and stretched towards the periphery. However, both surfaces do not yet form smooth areas and appear to be composed of rounded beadlike blastomeres.

Stage VI (10–11 hr uterine age; Figs. 11 and 12). The entire cytoplasmic mass of the germinal disc is cleaved, both on the upper and lower surfaces. The cells are very small and form an epithelial sheet of uniform thickness. The beadlike appearance of the individual cells is lost and the germ can legitimately be called a blastoderm.

Period B: Formation of Area Pellucida (Stages VII–X)

Stage VII (12–14 hr uterine age; Figs. 13 and 14). The cells of the upper surface became much smaller as a result of intensive dividing, although at the magnification used by us it is difficult to distinguish the individual cells. The cells of the lower surface are, however, much larger and generally similar in size to the cells of the lower surface of stages V–VI.

The epitheliumlike appearance of stage VI is modified as a result of the shedding of some cells from a limited area of the lower surface at the posterior half of the germ. The shed cells rest on top of the yolk at the bottom of the subgerminal cavity. The region that shed the lower cells forms a transparent thinned-out area in the posterior half of the germ and is the first sign of the area pellucida (a.p.). From this stage onward the diameter of the germ increases with progressive development.

Stage VIII (15–17 hr uterine age; Figs. 15 and 16). The transparent region has spread to both sides and forms a sicklelike area. It is now obvious that a marginal band of the germ, to become the area opaca (a.o.) is not included in the new process and does not change its appearance. From the ventral side, the still very large beadlike cells of the lower surface (y.l.c.) can be seen to lose contact and fall off.

Stage IX (17–19 hr uterine age; Figs. 17 and 18). The transparent region is now

FIG. 1. Upper surface of stage I germ demonstrating cleavage furrows (c. fur.) polar body (p.b.) nests of supernumerary spermia (su. sp.).

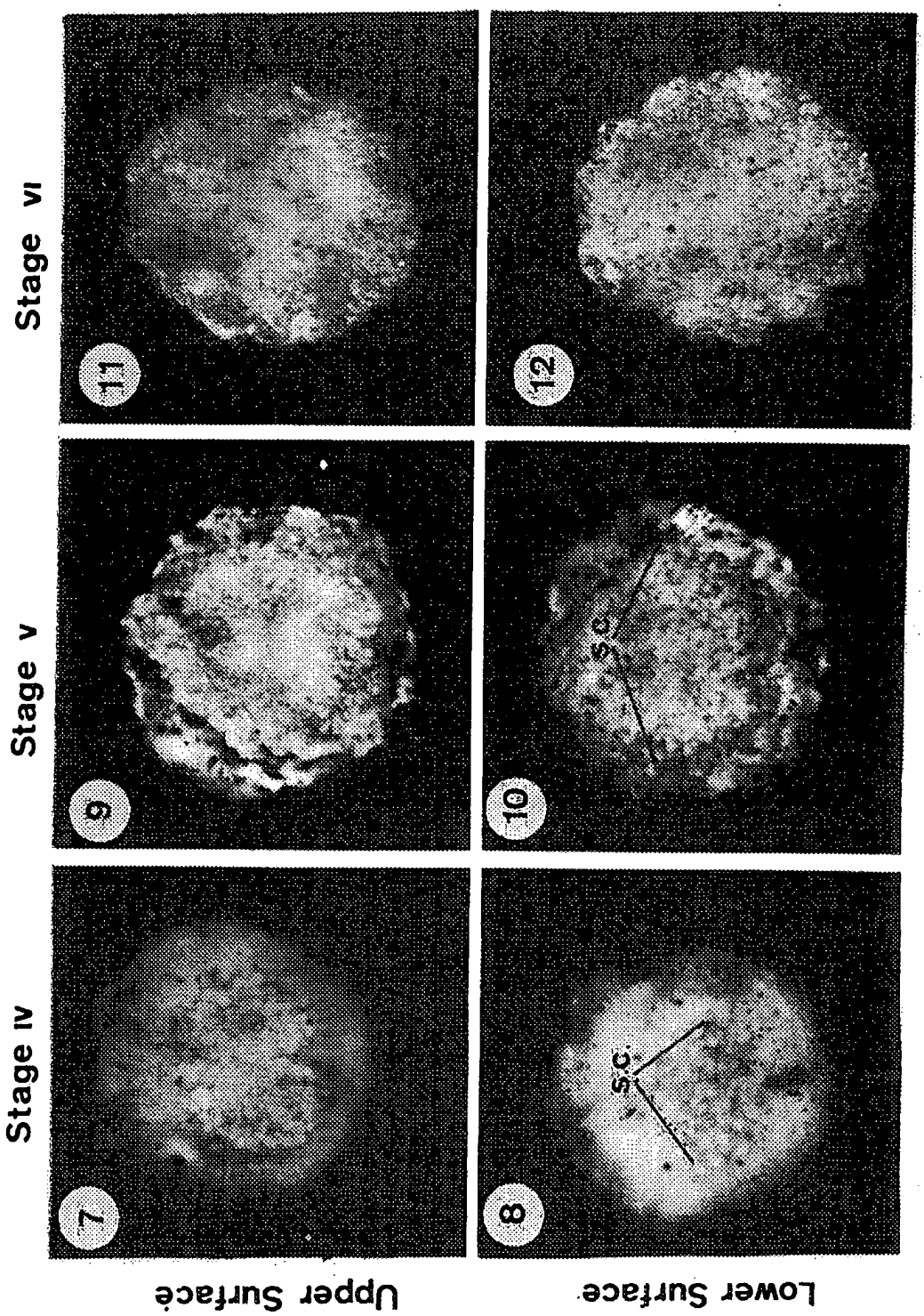
FIG. 2. Lower surface of same stage I germ. No cell boundaries discerned. Large vacuoles (vac.) randomly distributed.

FIG. 3. Upper surface of stage II germ. Cleavage has proceeded. More centrally located ones still open laterally.

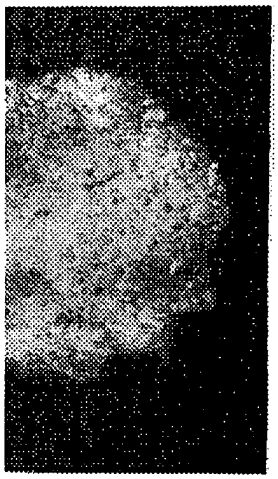
FIG. 4. Lower surface of same stage II germ. No change from stage I (Fig. 2) except for small vacuoles.

FIG. 5. Upper surface of stage III germ. Cleavage proceeds towards the periphery. Central blastomeres smaller than more laterally located ones.

FIG. 6. Lower surface of same stage III germ. At the center a few blastomeres are seen closed off also on their ventral side (cl. bl.). Vacuoles numerous, remarkably smaller, occupy area lateral to closed off blastomeres.



FIGS. 7-12.



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FIGS. 7-12.

spreading in an anterior direction, although the process is not yet completed and the border between the area opaca and the area pellucida is not yet a sharp one, especially at the anterior end.

Stage X (a freshly laid egg about 20 hr uterine age; Figs. 19 and 20). Observation both from the upper and lower surfaces reveals that the formation of the area pellucida has been completed and there is a clearly demarcated border between it and the area opaca. However, this stage not only terminates period B (the formation of the area pellucida), but is also the beginning of the following period C. On closely observing the lower surface, clusters of small cells (much smaller than the cells shed during the formation of the area pellucida) are seen to form a meshlike layer at the posterior area of the blastoderm i.ag.). Only the most posterior region of the area pellucida fails to be involved in this new process and remains as a transparent sickle shaped belt (t.b.).

Period C: Hypoblast Formation (Stages XI–XIV)

All the stages included in this period and onward are arrived at by incubating the freshly laid egg for varying lengths of time. Incubation time depends in each case on the initial stage of the embryo, which in turn depends on the time lapse since laying and on the environmental temperature. Although the ventral cell clusters of stage X may be regarded as the first signs of hypoblast formation, from a morphologic point of view, it is preferable to refer to an actual structure, which can be directly related to the hypoblast.

Stage XI (Figs. 21 and 22). Observation of the upper surface of the blastoderm reveals a smooth thin layer through which deeper concentrations of cells may be seen. The same picture, though slightly better defined, may be seen when looking at the ventral side of the blastoderm. Here again in front of the posterior section of the area opaca a transparent beltlike area (t.b.) may be seen, the anterior border of which is demarcated by a relatively narrow horseshoelike concentration of cell clusters (k.s.). Inside this horseshoe a number of individual clusters of various sizes may be seen (i.ag.). The cell clusters demonstrate a clear antero-posterior orientation with the bigger ones at the posterior side and the smaller ones situated more anteriorly. The horseshoelike concentration of cells at the posterior side of the blastoderm is probably what is referred to in the literature as Koller's sickle and from a morphologic point of view, the beginning of the hypoblast.

Stage XII (Figs. 23 and 24). Here again the transparent posterior belt may be seen, anterior to which the newly formed lower layer, the hypoblast, already lines half of the lower surface of the area pellucida. The hypoblast, although, sheetlike, is not entirely continuous and gives the impression of being formed by the fusion of separate cell masses.

Stage XIII (Figs. 25 and 26). The process of hypoblast formation has been completed. The posterior margin of the hypoblast is still very accentuated on the ventral side and also may be seen very clearly from the dorsal side through the transparent epiblast. The upper surface continues

FIG. 7. Upper surface of stage IV germ. Cleavage proceeds. Central cells becoming smaller; lateral cleavage furrows reach margin of germ.

FIG. 8. Lower surface of same stage IV germ. Amount of ventrally closed blastomeres increases. Beneath latter subgerminal cavity (s.c.) is expanding.

FIG. 9. Upper surface of stage V germ.

FIG. 10. Lower surface of same stage V germ. Almost all blastomeres closed ventrally and subgerminal cavity (s.c.) remarkably enlarged.

FIGS. 11, 12. Upper and lower surface views of same stage VI blastoderm. Fully cleaved, individual cells have lost beadlike form and are incorporated into epithelial sheet. Upper and lower surfaces appear similar.

FIGS. 13–18.

to be smooth without any sign of depression or invagination.

Stage XIV (Figs. 27 and 28). The anterior side of the hypoblast now also has well-defined borders and at the posterior side a cellular bridge is being formed between the hypoblast and the area opaca (pos. br.).

Hamburger–Hamilton stage 2 (Figs. 29 and 30). Here the primitive streak (p.s.) can be seen to form in the cellular bridge posterior to the initial posterior margin of the hypoblast.

DISCUSSION

Although much work has been done on early stages of chick development during the last 100 years, there is nevertheless confusion about development prior to primitive streak formation. To date, no systematic study has been made of this entire period, and the existing fragmentary data suffer from drawbacks. The main disadvantage of the sporadic data was that the material had been studied after fixation and generally only from microscopical sections. In those cases in which sectioning was attempted according to Von Baer's law (which is known to be inaccurate), a deviation of a few degrees might have lead to misinterpretations. Also, no criteria existed to arrange the data gathered from all the different observations in a correct temporal developmental order.

The aim of the present study was therefore to rectify the above-mentioned shortcomings by systematically studying one stage after another with an exact timing of the stages; by studying live material, photographing it as promptly as possible, and paying attention to the exact anterio-posterior orientation of the embryo. The present study deals only with the information gained by using a stereoscopic microscope; however, parallel material that was collected and processed for both light and electron microscopy has been studied and will be discussed separately.

The developmental period least affected by the above-mentioned drawbacks is the cleavage period. Here, even without timing, there can be no doubt about the order of the stages. The studies of both Patterson (1910) and Olsen (1942) provide quite accurate correlation between the time that elapsed from the laying of the previous egg and the number of blastomeres seen in surface view. Neither of these authors, however, attempted to stage the germs of the period studied, to photograph them in a live condition, nor to observe the germ's lower surface while checking its relation to a subgerminal cavity. Patterson even mentions that in the hen's egg, photographs of the living cells are impossible, this in contrast to the pigeon's egg in which the cleavage furrows stand out clearly; the early stages of the latter were successfully photographed by Blount (1909). Probably, it was Patterson's statement that discouraged later investigators from attempting to deal with the living germ. Olsen (1942) also fixed the embryo and stained it with gentian violet to accentuate the cleavage furrows. In the present study, it was found that if a living germ is removed from the yolk it can be photographed on a black background when correctly illuminated.

FIG. 13. Upper surface of stage VII blastoderm. Upper surface remains smooth, but its posterior half becoming transparent, indicating formation of area pellucida (a.p.).

FIG. 14. Lower surface of same stage VII blastoderm. Area pellucida formed through process of cell dropping.

FIG. 15. Upper surface of stage VIII blastoderm. Area pellucida expands from original posterior region in anterio-lateral direction. Marginal area, the area opaca (a.o.), remains unaffected by this process.

FIG. 16. Lower surface of same stage VIII blastoderm. Shedding off of ventral yolk laden cells (y.l.c.) is clearly seen.

FIGS. 17, 18. Upper and lower surface of same stage IX blastoderm. Formation of area pellucida nearly completed.

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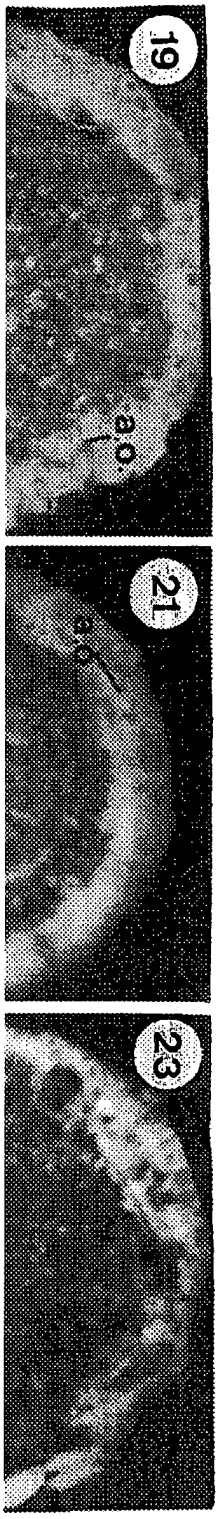


Not only are the furrows visible, but in certain cases also the polar bodies (p.b.) and the supernumerary spermia (su. sp.), exactly in the same way as in the pigeon (Blount, 1909). By observing the first cleavages in many germs (Figs. 31–36) it may be seen that cleavage can start at any point in the germ, that the first furrow which is often eccentric does not divide the germ into two equal parts, and therefore, has nothing to do with the future axis. This is in accordance with many earlier observations (Duval, 1884; Patterson, 1910; Olsen, 1942). As far as the synchrony of the cleavages is concerned, there is also disagreement among different investigators. A recent report by Emanuelsson (1965) claims that the first five divisions, that is, up to 32 blastomeres, are synchronous, whereas earlier investigators claim that the third cleavage is already irregular (Patterson, 1910; Blount, 1909). Our results agree with the findings of Patterson and Blount, whose observations were based on a much more extensive series of embryos. Generally, it may be stated that in most cases the third cleavage, and sometimes the second cleavage, are already asynchronous and irregular, although occasionally regular cleavages also may be found (Figs. 35 and 36). Another feature that is very prominent in live, cleaving germs is the abundance of vacuoles which are large in the earliest stages and become progressively smaller until they disappear altogether at stage VI, the end of cleavage. Most investigators do not mention the vacuoles, and here again attention was paid to them by workers such as Disse (1878), who disputes with His (1968) about the latter's belief that there is a progressive accumulation of vacuoles that later contribute to the formation of the subgerminal cavity. Also, Goette (1874) and Oellacher (1869) attributed the formation of the subgerminal cavity to the accumulation of fluid. Disse experimented with fixation and concluded that the formation of vacuoles is not physiologic, but an artifact caused by the chromic acid. All the above investigators neglected looking at a nonfixed embryo which readily demonstrates an abundance of vacuoles. Although we cannot yet say anything concerning the connection between the vacuoles and the subgerminal cavity, we can state that such a cavity filled with fluid does exist. Here again, there is in the literature a discussion based on microscopical slides on whether the cavity, seen by many investigators, is real or an artifact. Jacobson (1938) and Pasteels (1945) believe that there is only a narrow slit between the germ and the underlying yolk, the cavity seen in the slides being artifactual. Clavert (1960, 1962), who based his conclusions on observations of live material, but of the intact egg without trying to remove the germ from the underlying yolk, is even more radical. He believes that only 6–8 hr before the egg is laid (our stages VII–VIII) "the germ detaches itself from vitellus . . . the area pellucida then appears, off center." Our material demonstrates that the subgerminal cavity exists from stage III onward. It begins in the region where the first cells close off ventrally, as a very small centrally situated cavity, and it then extends gradually towards the periphery to spread under the entire germ at stage

FIGS. 19, 20. Upper and lower surface of same stage X blastoderm. On lower surface of thinned out blastoderm isolated cell aggregates (i. ag.) appear, more concentrated at posterior half. Transparent belt (t.b.), however, separates aggregates from area opaca (a.o.).

FIGS. 21, 22. Upper and lower surface of same stage XI blastoderm. Most posterior aggregates coalesce to form Koller's sickle, anterior to transparent belt.

FIGS. 23, 24. Upper and lower surface of same stage XII blastoderm. Koller's sickle being its posterior margin, a hypoblast (hyp.) is forming progressing in an anterior direction. At this stage hypoblast underlies only posterior half of the epiblast.

FIGS. 25–30.

VI. This cavity is filled with fluid and can be penetrated easily with a needle during the operation.

This brings us to consider the true meaning of the appearance of the area pellucida (a.p.). The formation of the a.p. was a neglected period in the chick's development probably because it is intrauterine and because it is relatively short and takes 4–6 hr. Most of the investigators did not even realize that this period is a very critical one in development and were concerned only with the existence of the underlying cavity. It was Clavert (1960, 1962) who stressed the connection between the appearance of the a.p. and the attaining of bilateral symmetry by the germ. Clavert realized that he could change the orientation of the embryo by rotation in different directions, but this was effective only "as long as the area pellucida has not begun to appear. The formation of the a.p. corresponds with some important transformation."

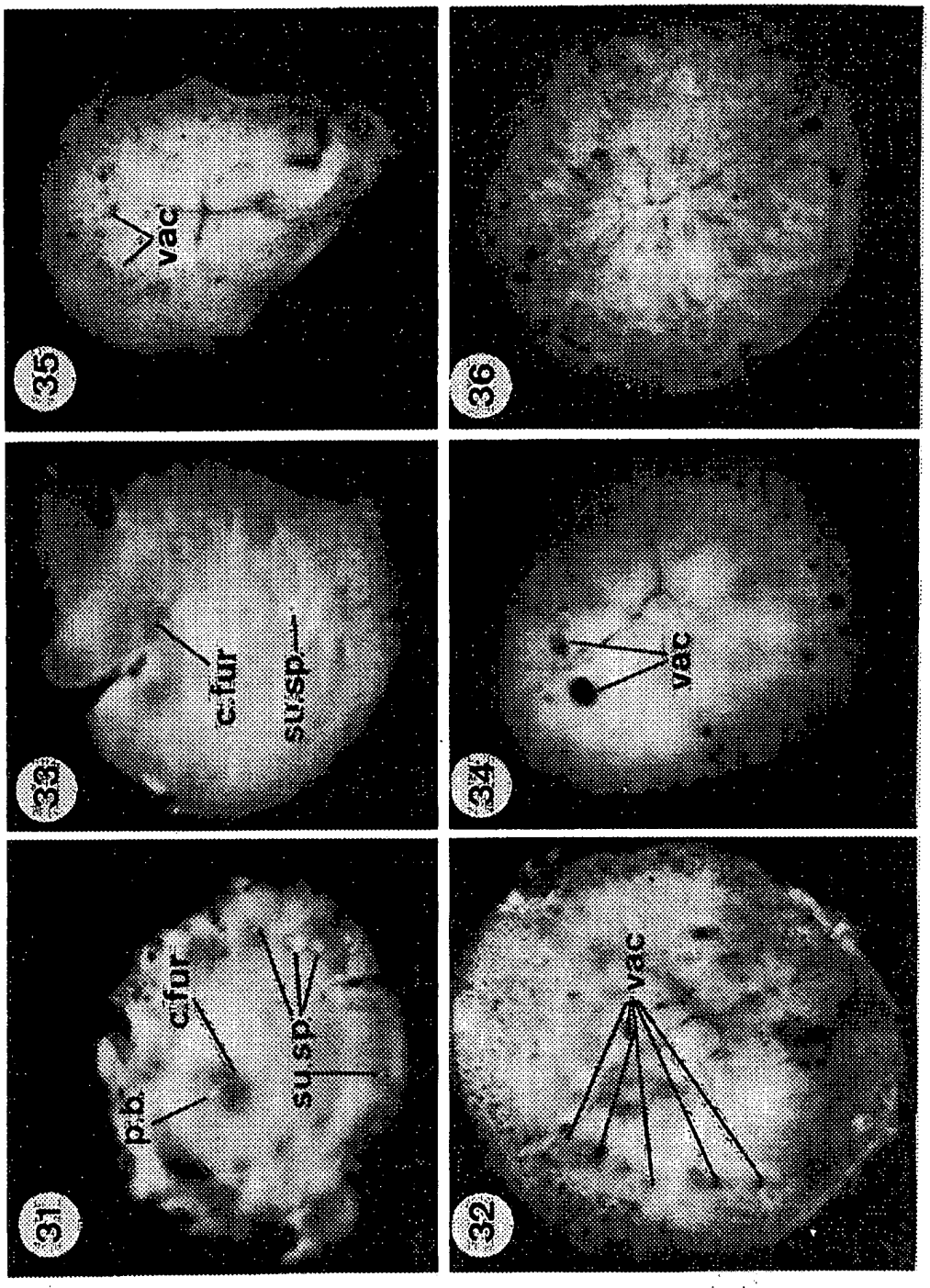
By trying to analyze the phenomenon of symmetrization (Kochav and Eyal-Giladi, 1971) we found that it was not rotation but the force of gravity that induced symmetry in the germ. By hanging the yolks of stage VI uterine eggs by their chalazae in beakers filled with Ringer's solution we were able to observe the process of symmetrization, and we saw that the a.p. began to appear at the future posterior side of the germ by the appearance of a transparent area, which gradually extended in an anterior direction. Up to this point we agree with the descriptions of Patterson (1909) for the pigeon and Clavert for the chick, but from here on our interpretations part. Clavert interpreted the appearance of the transparent spot as a "brutal detachment of the germ from the vitellus," which we have now seen to be incorrect. He also agrees with Kolliker (1875) that at the same time a rearrangement of cells occurs so that the germ that was five to six cells thick in its central region, is by then two to three cells thick. The answer to what is going on during the appearance of the transparent a.p. lies again in the observation of the lower surface of the live germ. From stage VII to stage X something brutal happens to the germ, but not a detachment from the yolk. Instead, many cells detach themselves in an orderly way from the ventral side of the multilayered opaque germ, to transform the germ by a gradual process, progressing from the future posterior to the future anterior side, into a thin transparent germ. The shed cells are found in the subgerminal cavity in the form of yolk balls (Dotter-Kugeln of Goette, 1874; Peter, 1938; or Furchungskugeln of Kolliker, 1875), the existence of which could not be explained adequately until now. We describe here the first morphogenetic happening in chick development during which the direction of the embryonic axis is not only determined, but also can be seen very clearly.

We are dealing with two quite different phenomena which have thus far been confused in the literature. The formation of the subgerminal cavity is connected with the closing off of the lower side of the ventrally open blastomeres. This process therefore starts somewhere in the central region of the germ and spreads gradually centrifugally. At the end of stage VI, the cavity is spread under the entire germ and is a real gap filled with fluid. After the

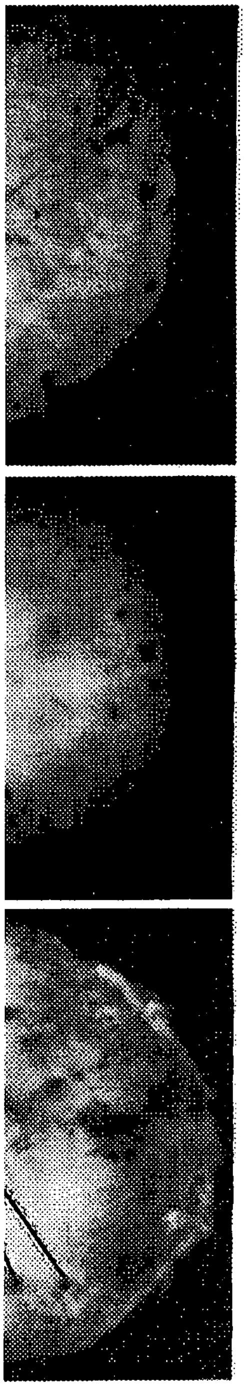
FIGS. 25, 26. Upper and lower surface of stage XIII blastoderm. Full hypoblast (hyp.) is formed. Koller's sickle (k.s.) is still visible at posterior margin. Transparent belt (t.b.) now surrounding area occupied by the hypoblast separating it from area opaca (a.o.).

FIGS. 27, 28. Upper and lower surface of stage XIV blastoderm. At posterior side of blastoderm cellular bridge (pos. br.) develops connecting hypoblast with area opaca.

FIGS. 29, 30. Upper and lower surface of Hamburger and Hamilton stage 2 blastoderm. Posterior bridge is clearly seen and in it beginning of primitive streak (p.s.).



FIGS. 31–36. Six blastoderms of stage I demonstrating different patterns of early cleavage. c. fur.



FIGS. 31–36. Six blastoderms of stage I demonstrating different patterns of early cleavage. c. fur.,

formation of the cavity is completed, a morphogenetic process, started by the force of gravity, begins in an oriented posterio-anterior direction. This morphogenetic process is externally expressed by the orderly shedding of the lower layers of cells starting at the future posterior and ending at the future anterior side, leaving a denuded central area as a thin transparent sheet—the area pellucida. At the termination of the above-described process, the symmetrized egg is ready to be laid. In the freshly laid egg, two distinct circular areas can already be distinguished, the peripheral area opaca and the central very thin, essentially single-layered area pellucida. By now the symmetrized germ looks radial again and it is difficult for an inexperienced eye to determine its polarity. However very few investigators have faced this problem at the above stage of development (stage X). To get embryos at stage X one usually has to collect the eggs immediately on being laid, remove the germ from the yolk without delay, and inspect its lower surface. Unincubated eggs that are left for a few hours or more at room temperature, continue to develop slowly and when opened are found to be in a much later stage. As most investigators have the eggs supplied to them, they are received at a relatively late developmental stage, which accounts for the numerous faulty descriptions of an unincubated germ. Bellairs (1971) summarizes the literature on this point by saying that "hens' eggs have generally become two-layered" when the egg is laid.

The question of how the germ becomes two-layered has been dealt with by many investigators, most of whom again base their studies on fixed and sectioned material. The literature on this issue is absolutely fascinating, as almost every possible explanation for the formation of the hypoblast has been adopted by somebody. There is at least a general concensus that the germ was once, while inside the uterus, a single-layered embryo and then became double layered. Balfour (1878) believed that the large loose yolk balls (our yolk-laden cells) found at the bottom of the subgerminal cavity, which he named formative cells after Peremeschko (1868), suddenly begin to undergo a process of metamorphosis; from being spherical and non-nucleated, they become flattened and nucleated. These cells form hypoblast first at the center and later at the circumference. Disse (1878) prefers the idea of a gradual formation of the hypoblast from the periphery towards the center in a concentric fashion. Koller (1882) and Duval (1884), followed by Patterson (1909) for the pigeon, Jacobson (1938) for the chick, and Lutz (1955) for the duck, were very anxious to find a homology between the process of hypoblast formation and gastrulation in amphibians and managed to find each in his own way a blastopore either somewhere in the embryonic disc or its posterior margin.

A quite different approach was initially taken by Oellacher (1869), extensively developed by Peter (1939), and later supported by Pasteels (1945), who after studying the duck blastoderm changed his mind about his earlier observation of the chick (1937). They claim that "in the single layered germ clefts appear, progressively separating a superficial continuous epithelium from deeper cells." This process called by them delamination, results in the formation of a bilaminar embryo.

Chen (1932) interpreted the formation of the primary hypoblast in the duck as a forward growth by proliferation of the thickened posterior germ wall. He, however, mentioned that the entoderm in the unincubated blastoderm is a loose layer of separate cells, and that it becomes filled in, to form a continuous coherent sheet during early incubation.

Many other investigators (His, 1868; Nowack, 1902; Merbach, 1935; Pasteels, 1937; Vakaet, 1962; Spratt and Haas, 1965) have noticed that the primary hypoblast starts as a noncoherent layer, especially in the

future anterior region, and interpreted its appearance as a process of "polyinvagination." Some of the above-mentioned authors added other mechanisms to the polyinvagination. For instance, Merbach (1935) advocated in addition an invagination at the posterior end.

Others, like Nowack (1902), Vakaet (1962), and Spratt and Haas (1965), claim that in addition to polyinvagination there is an anteriorly directed movement of cells of the lower layer from a posterior concentration of cells. Of the last group of authors, Nowack denies the existence of a posteriorly situated concentration of cells (Koller's sickle) from which the initial primary hypoblast grows anteriorly, whereas Vakaet (1962) and Spratt and Haas (1965) agree that a concentration of cells reminiscent of Koller's sickle exists, although there is probably no furrow (Sichelrinne) to account for a real invagination (blastopore), as suggested by Koller (1882) himself.

The material presented here demonstrates very clearly all the stages of formation of the primary hypoblast. At stage X, while the area pellucida is completed, the primary hypoblast is already starting to form as isolated clusters (i.ag.) of cells being more crowded at the posterior side of the germ. However, there is a transparent beltlike, pure epiblastic region (t.b.) separating the above aggregates from the posterior area opaca. At stage XI, there is a notable increase in cell population density at the posterior side forming a clearly demarcated sickle (Koller's sickle, k.s.), anterior to which the as-yet noncoherent cell clusters also seem to grow in size. At stage XII, the sickle becomes coherent with the aggregates of the posterior half to form the posterior region of the primary hypoblast, probably the area called by Koller (1882) the embryonic shield and by Peter (1938) the Entodermhof. Also at this stage the posterior sickle and the transparent belt behind it are well defined. The same is also true for stage XIII, at which the hypoblast has been completed. We are confronted with a postero-anteriorly directed second morphogenetic event, following very closely in time and orientation the formation of the area pellucida. Whereas the formation of the area pellucida is characterized by the scattered appearance (polyinvagination) of isolated cells and cell aggregates on the lower surface of the germ. These aggregates coalesce in an orderly manner to form the gradually growing primary hypoblast sheet.

We do not as yet have sufficient data to decide whether the formation of the hypoblast involves only a coalescence of the isolated aggregates or also an active anteriorly directed movement.

Toward the end of hypoblast formation, a cellular bridge appears connecting the central region of the posterior sickle with the posterior area opaca (Figs. 27 and 28, pos. br.). Inside this bridge, the first rudiment of the primitive streak appears. It is not yet clear how this bridge is formed.

After the complete picture of the gross morphologic events has been clarified it is felt that a revision of some terms currently used for the early stages is necessary. The confusion is best demonstrated in textbooks: Patten (1971) uses the term blastodisc for the cytoplasmic disc before cleavage, whereas a blastoderm is a germ after many divisions. Balinsky (1970) inconsistently uses the terms blastoderm and blastodisc for the same developmental stages. Bellairs (1971) does not use the term blastodisc at all and mentions only the term blastoderm. It is therefore suggested that the term germ be employed for the developing chick embryo. The term blastodisc should be avoided, and if desired a germ from stage VI onwards can be called a blastoderm.

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Meeting Info.: Nineteenth Annual Meeting of the Southern Poultry Science Society and the 39th Annual Meeting of the Southern Conference on Avian Diseases Atlanta, Georgia, USA January 19-20, 1998
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